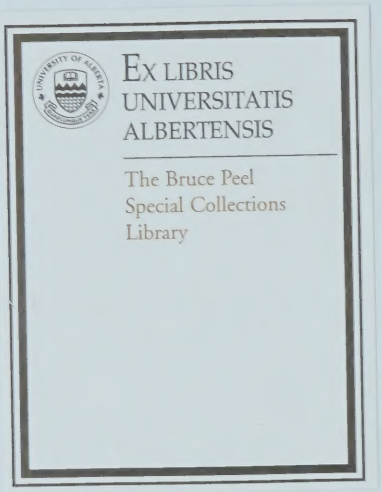
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Characterization and Regulation of Triacylglycerol Hydrolase

by

Dean Gilham

A thesis submitted to the Faculty of Graduate Studies and Research

in partial fulfillment of the

requirements for the degree of Doctor of Philosophy

Department of Cell Biology

Edmonton, Alberta
Fall 2005

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled "Characterization and Regulation of Triacylglycerol Hydrolase" submitted by Dean Gilham in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Date Thesis Approved

DEDICATION

To Bardy Gilham, Trenna Gilham and Tracey Lamoureux

Thank you for your unconditional support and belief

in me through my life, and especially over the years in university.

To my wife, Bonnie Gilham

Thank you for the love and understanding you have demonstrated over and over.

Thank you also for bringing us our two wonderful children;

you are the loves of my life.

ABSTRACT

Very low density lipoproteins (VLDL) are large complex particles comprised of a variety of lipid and protein components. VLDL is assembled by the liver and secreted into the bloodstream. The most abundant lipid component of VLDL is triacylglycerol (TG). VLDL-TG is largely derived from hepatic TG storage pools rather than from *de novo* synthesis. The recruitment of stored TG for assembly onto VLDL involves lipolysis followed by re-esterification in the endoplasmic reticulum (ER). Triacylglycerol hydrolase (TGH) is a lipase that mediates the lipolysis of stored hepatic TG. The protein has the most sequence identity with the carboxylesterase family of enzymes. Chemical inhibition of TGH in intact cells reduced the secretion of TG and of the main protein component of VLDL, apolipoprotein (apo) B. The subcellular distribution of the enzyme appears to be critical for its efficient participation in VLDL assembly. TGH is a soluble protein retained in the lumen of the ER by an unusual carboxy-terminal tetrapeptide, -HIEL. Mutation of this retention sequence to the consensus for ER retention, -KDEL, results in altered sub-ER distribution of TGH and in decreased ability of the enzyme to mobilize stored TG for secretion. This is the first demonstration of a sequence influencing distribution of a protein within the lumen of this organelle. Investigations on how post-translational modifications may regulate TGH function were performed. TGH is a glycoprotein, however, glycosylation is not required for folding or enzyme activity. Many lipases are modified by phosphorylation, inducing a variety of functional changes; TGH, however, is not modified in this manner. TGH associates with lipids, but this

interaction is not mediated through acylation of its one free cysteine residue. Developing VLDL particles co-exist with TGH in the ER, however, apoB associated lipids are not substrates for TGH. Therefore, delipidation of secretion-incompetent apoB-containing particles destined for degradation is not mediated by TGH. Evidence that TGH interacts with other proteins is provided; the future identification of TGH interacting proteins may clarify the molecular mechanism of VLDL assembly.

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LIST OF ABBREVIATIONS

ACAT	acyl-coenzyme A:cholesterol acyltransferase
ADRP	adipocyte differentiation related protein
AGPAT	1-acylglycerol-3-phosphate acyltransferase
ATGL	adipose triglyceride lipase
apo	apolipoprotein
BSDL	bile salt dependant lipase
BiP	endoplasmic reticulum binding protein
BSA	bovine serum albumin
cAMP	cyclic adenosine monophoshate
CCT	cytidine triphosphate: phosphocholine cytidylyltransferase
cDNA	complementary deoxyribonucleic acid
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CE	cholesteryl ester
CoA	coenzyme A
CVD	cardiovascular disease
DG	diacylglycerol
DGAT	diacylglycerol acyltransferase
DGTA	diacylglycerol transacylase
DMEM	Dulbecco's modified Eagle's medium
DTBP	dimethyl 3,3´-dithiobispropionimidate
E600	diethyl-*p*-nitrophenyl phosphate
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(2-aminoethylether)-tetraacetic acid
ER	endoplasmic reticulum
Es 22	esterase 22/egasyn
Es X	esterase X
FBS	fetal bovine serum
GPAT	glycerol-3-phosphate acyltransferase
HDL	high density lipoprotein

HS	horse serum
HSL	hormone sensitive lipase
IP	immunoprecipitation
LDH	lactate dehydrogenase
LDL	low density lipoprotein
LpL	lipoprotein lipase
MAP kinase	mitogen-activated protein kinase
MEM	Minimum Essential Medium
MG	monoacylglycerol
MGAT	monoacylglycerol acyltransferase
mRNA	messenger ribonucleic acid
MTP	microsomal triglyceride transfer protein
MUH	4-methylumbelliferyl heptanoate
PAGE	polyacrylamide gel electrophoresis
PC	phosphatidylcholine
PEMT	phosphatidylethanolamine N-methyltransferase
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDI	protein disulfide isomerase
PEMT	phosphatidylethanolamine *N*-methyltransferase
PAPH	phosphatidic acid phosphohydrolase
PKA	cyclic adenosine monophospate dependant protein kinase
PPAR	peroxisomal proliferator activated receptor
RT-PCR	reverse transcriptase PCR
SCAP	SREBP cleavage-activating protein
SCD	stearoyl-CoA desaturase
SDS	sodium dodecyl sulphate
SREBP	sterol regulatory element-binding protein
TBS	Tris buffered saline
TG	triacylglycerol
TGH	triacylglycerol hydrolase

TLC	thin layer chromatography
TZD	thiazolinedione
VLDL	very low density lipoprotein

Chapter 1:

Introduction

Portions of this chapter have been adapted from:

1) Dolinsky, V.W., D. Gilham, M. Alam, D.E. Vance, and R. Lehner. (2004b) Triacylglycerol hydrolase: role in intracellular lipid metabolism. *Cell. Mol. Life Sci.* 61:1633-1651.
2) Gilham, D., and R. Lehner. (2004) The physiological role of triacylglycerol hydrolase in lipid metabolism. *Rev. Endocr. Metab. Disord.* 5:303-309.

The adaptations and any figures from these publications have been incorporated in this Chapter with permission from the respective journals.

1.1 Triacylglycerol and human disease

1.1.1 Triacylglycerol metabolism is linked to several human diseases

The ability to store neutral lipids plays a critical role in the ability of an organism to withstand fuel deprivation. Triacylglycerol (TG) is the most concentrated form of energy available to biological tissues. It can provide over double the amount of energy by metabolic oxidation than an equal mass of either carbohydrates or proteins (Brindley, 1991). TG is composed of a glycerol backbone to which three fatty acids are attached via ester bonds (Figure 1-1). Most cells and tissues have the ability to synthesize and store TG to some degree. TG metabolism is of tremendous clinical relevance. Dysregulation of neutral lipid synthesis, transport or storage contributes to the development of disease. Consequently there is tremendous clinical and pharmacological relevance for enzymes that metabolize neutral lipids. Obesity alone is associated with type 2 diabetes, hyperlipidemia, cardiovascular disease, stroke, gallbladder disease and some cancers (AHA, 2004; Husten, 1998a; NIH, 1998; Ross, 1993; WHO, 1998; Zimmet *et al.*, 2001). Excessive TG storage within muscle and pancreatic β-cells correlates with insulin resistance, leading ultimately to diabetes (Kahn and Flier, 2000; Shulman, 2000). Excessive hepatic TG secretion contributes to high levels of circulating TG in the blood (Castelli, 1986), which is a risk factor for cardiovascular disease (CVD). Plasma TG levels >150 mg/dL or 1.70 mM in a fasted state are considered elevated (NCEP, 2001). Therefore, excessive levels of tissue or circulating TGs have negative effects on health. If the balance between TG synthesis and storage versus TG hydrolysis and fatty acid oxidation is disturbed, the development of obesity, atherosclerosis and/or diabetes is likely. A more robust understanding of TG metabolism at the molecular level is essential if we are to develop prevention strategies and treatments for lipid related diseases.

Within this introduction, how lipids contribute to disease will be described, and then the focus will narrow to a particular enzyme involved in TG metabolism know as triacylglycerol hydrolase (TGH). A review of this enzyme in the context of a general discussion of lipid metabolism will entail the remainder of this

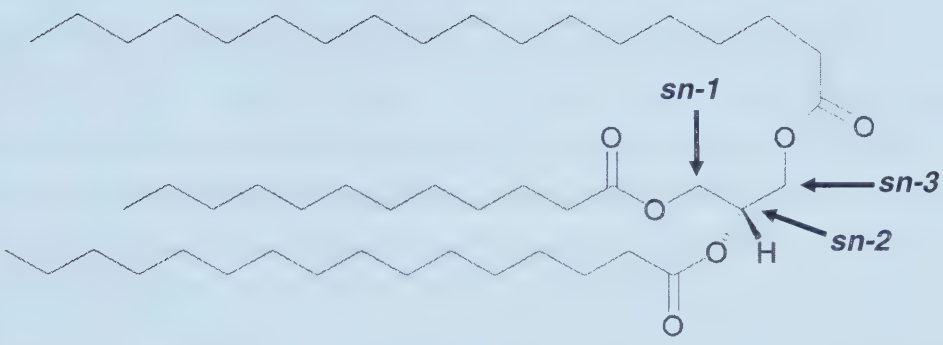

Figure 1-1 Representation of the chemical structure of triacylglycerol. Triacylglycerol is formed when three fatty acids are bound to a glycerol molecule via ester bonds. The three carbon atoms of the glycerol backbone are indicated with arrows, as well as their stereospecific numbering (*sn*). Triacylglycerol is a very efficient energy storage molecule. Because it is hydrophobic in nature, storage of TG does not affect the osmotic pressure of the cell. As shown, the hydrocarbon chain may be of various lengths. Further variation of this class of molecules is provided by introducing double bonds in the acyl chains.

Chapter. Experiments that advance our knowledge of TGH, as well as foreseeable future directions, are presented in the following Chapters of this Thesis.

1.1.2 Obesity trends in North America

Several excellent reviews regarding lipid storage have been composed (Gibbons *et al.*, 2000; Murphy, 2001; Murphy and Vance, 1999). Storage of TG occurs when total energy intake exceeds total body energy expenditure. Excessive storage is evidenced by the all too prevalent condition of obesity. The sedentary lifestyle and high fat diet of the Western world have resulted in alarming increases in the occurrence of obesity. Unfortunately, the trend of increasing prevalence of obesity in Canada is obvious (Figure 1-2) (Katzmarzyk, 2002), with data demonstrating equally alarming trends from the USA (Figure 1-3) (Mokdad *et al.*, 2001; Mokdad *et al.*, 1999). Obesity may be defined as a state of increased body weight, more specifically of adipose tissue, to a magnitude sufficient to induce adverse metabolic consequences.

Functionally and clinically overweight and obese states are estimated via body mass index (BMI), which is calculated by body weight (in kilograms) divided by the square of the individual's height in meters (Wyatt, 2003). In 1998, the World Health Organization and the National Institutes of Health USA defined overweight as a BMI of 25-29.9 and obesity as a BMI of ≥30 (NIH, 1998; WHO, 1998). Although BMI is not accurate for predicting body fat for all body structures and can be particularly misleading in athletes, it is an essentially cost-free measurement, noninvasive and accepted as a reasonable method of assessment. Waist circumference measurements when combined with BMI further reduce the number of individuals that are misclassified. Non-obese waist circumference guidelines are <35 inches for women and <40 inches for men (Lemieux *et al.*, 1996; NIH, 2000).

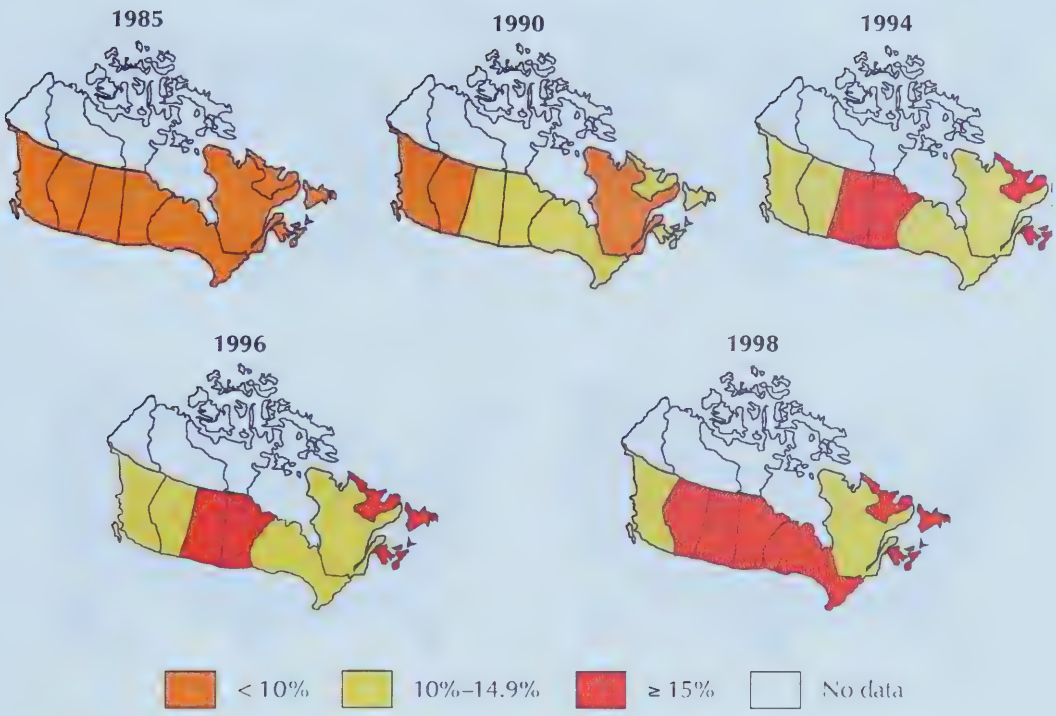

Figure 1-2. Prevalence of obesity amongst Canadian adults. In Canada, there is a trend towards an increasing occurrence of obesity. Because obesity is associated with a number of other serious disorders, there is concern for the overall health of Canadians. The data presented were obtained from self-reported heights and weights from Health Promotion Surveys and National Population Health Surveys. An obese state was then assigned from the calculation of body mass index as ≥30 kg/m^2. The figure was obtained from P.T. Katzmarzyk (2002) *CMAJ* 166:1039-1040 and is presented here with permission.

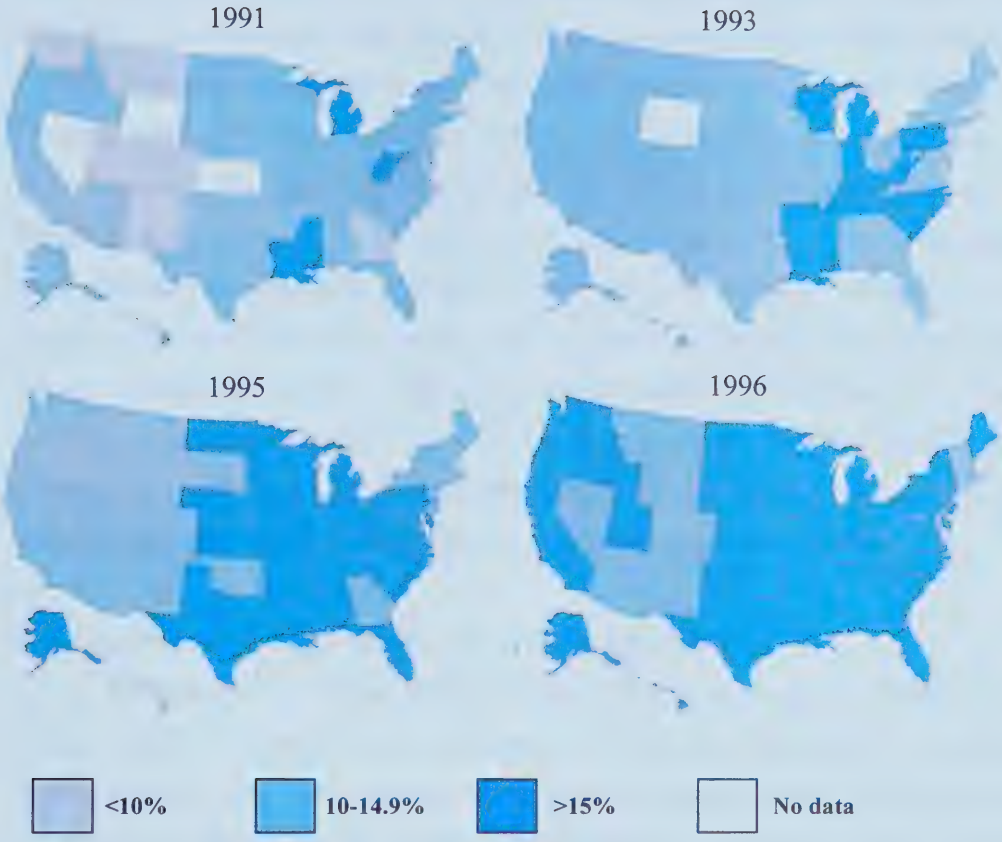

Figure 1-3 Prevalence of obesity amongst adults in the USA. The occurrence of obesity is increasing in the USA as it is in Canada. The trend suggests obesity related diseases will also increase. Data were calculated using the Behavioral Risk Factor Surveillance System, which is a telephone survey of non-institutionalized adults conducted by the Centers for Disease Control and Prevention and state health departments. States shown in white did not have data available. Obesity was defined by a body mass index of ≥30 kg/m^2. The figure was obtained from A.H. Mokdad *et al.* (1999) *JAMA* 282:1519-1522 and is presented here with permission.

1.1.3 Atherosclerosis and cardiovascular disease

The risk of death from CVD within industrialized nations is greater by far than the risk from any other cause. Most commonly, the underlying cause of CVD is the process of atherosclerosis (hardening of the arteries) (AHA, 2004; Husten, 1998a; Murray and Lopez, 1997). Atherosclerosis is the principal cause of heart attack and stroke in the Western world and is ultimately responsible for nearly half the mortality in the USA (AHA, 2004; Ross, 1993). Atherosclerosis is characterized by deposits (plaques) laden with cholesterol, cholesteryl esters (CE) and other lipids in the artery wall. It usually occurs in large and medium-sized arteries at branch points and bifurcations. While we now have a preliminary understanding of the pathogenesis and development of CVD, further advance is required for successful efforts towards decreasing the huge burden of lives lost and the economic cost surrounding this class of diseases.

1.1.4 Composition of the artery wall and atherosclerosis

As indicated in Figure 1-4, the artery wall is composed of distinct layers (Ross, 1986). The innermost layer, which is in contact with blood, is a monolayer of endothelial cells. Beneath this is the subendothelial space or intima, followed by the muscular layer or media, and finally the outermost layer called the adventitia. Atherosclerosis is evidenced by the presence of lesions affecting the endothelium, with macrophage and smooth muscle cell proliferation in the intima (Ross, 1995). As indicated above, the lesion area is heavily laden with cholesterol and CE, and continued lipid deposition in this area will decrease the elasticity of the vessel in that region and eventually occlude the arterial lumen. Worse is the rupture of the lesion, often drastically damaging the artery and spawning a blood clot that is the foundation of a life threatening thrombosis. The primary source of lipids for the pathogenic accumulation found in atherosclerosis is a particular class of fatty particles in the blood known collectively as lipoproteins.

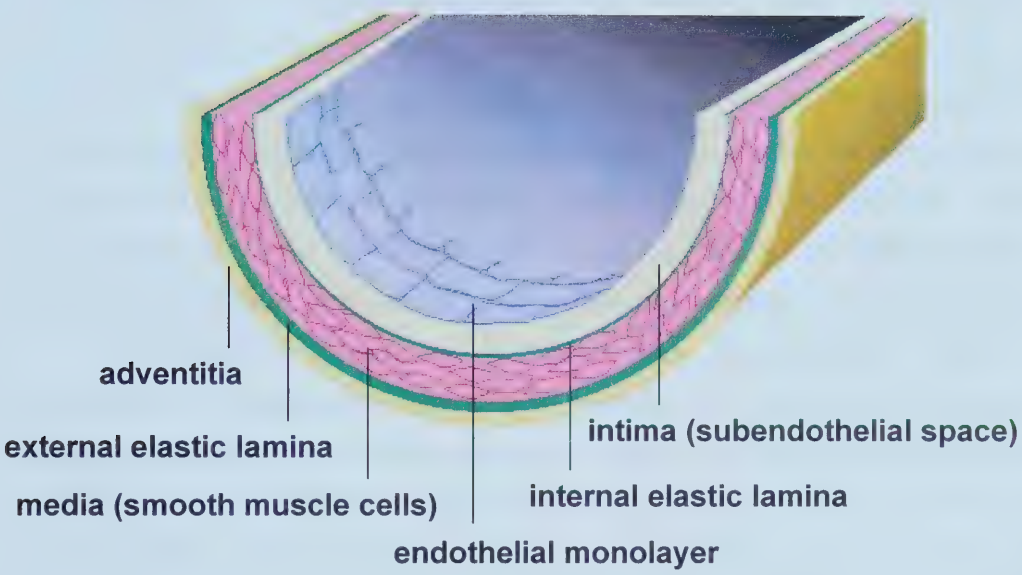

Figure 1-4 Layers of the artery wall. The endothelium, which is in contact with blood, overlays the intima, or subendothelial space. Beneath this is the internal elastic lamina followed by the media, a layer of smooth muscle cells. Underneath the media are the external elastic lamina and the outermost layer called the adventitia. The events of atherosclerosis mainly cause changes to the endothelium, the intima and the media.

1.1.5 Components and classification of lipoproteins

Neutral lipids like TG and CE are hydrophobic in nature, and hence have very low solubility in aqueous environments including blood. Therefore, in order to be transported throughout the body, where they are required for various cellular functions, hydrophobic lipids are packaged into complexes with specific proteins known as apolipoproteins as well as partly polar phospholipids. As shown in Figure 1-5, all mature lipoproteins consist of a hydrophobic core of nonpolar lipids including cholesteryl esters (CE) and TG surrounded by a phospholipid monolayer. The resulting complexes are termed lipoproteins, and are carried in the blood to deliver TG, phospholipids, cholesterol and other lipids to cells where they are utilized. There are a number of different classes of lipoproteins, which differ in size, composition and function as described below.

The amphipathic nature of phospholipids allows them to orient on the surface of the particle such that their hydrophilic head groups are exposed to the surface and their hydrophobic fatty acyl chains are directed towards the inner core. Free or unesterified cholesterol can also exist within the monolayer of phospholipid, allowing exposure of its free hydroxyl group to the surface. Apolipoproteins are also amphipathic and confer solubility to the lipoprotein particle as well as influence trafficking via interactions with various cell surface receptors, hydrolytic enzymes and lipid transferring proteins (Mahley *et al.*, 1984). Most apolipoproteins are exchangeable; they can detach from the surface of a lipoprotein then reattach to another. Hence the complement of apolipoproteins changes during the lifetime of these particles. The exception to apolipoproteins that exchange is the more hydrophobic and very large apolipoprotein (apo) B, which is integral and remains bound to the particle it is initially assembled with (Chan, 1992; Elovson *et al.*, 1988).

Lipoproteins are classified based on their density, which is determined by their lipid and protein composition and is inversely proportional to their diameter. Chylomicrons are the largest and least dense class of lipoproteins, with a very TG-rich core (75-1200 nm in diameter (Davis and Vance, 1996)). They are synthesized by the small intestine utilizing ingested dietary lipids, and are carried

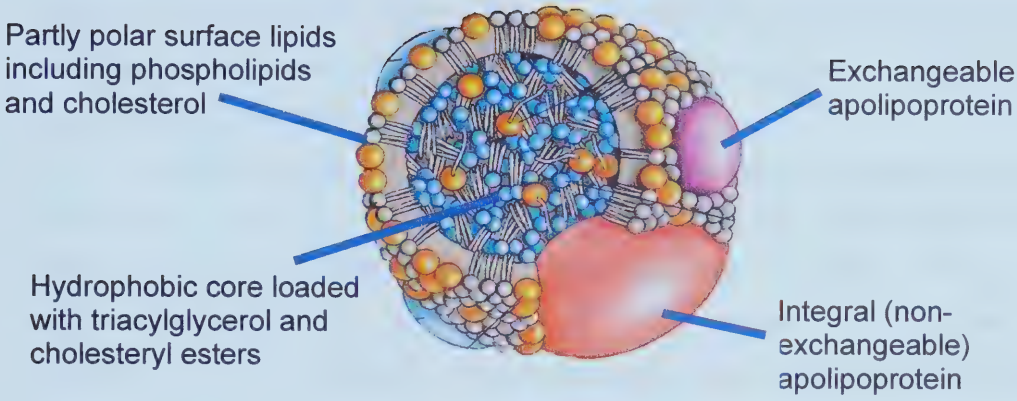
Partly polar surface lipids including phospholipids and cholesterol
Exchangeable apolipoprotein
Hydrophobic core loaded with triacylglycerol and cholesteryl esters
Integral (non-exchangeable) apolipoprotein

Figure 1-5 Generalized structure of a typical lipoprotein. The outer monolayer consists of amphipathic phospholipids and cholesterol. Thus lipoproteins are soluble in aqueous environments. The hydrophobic core is composed of triacylglycerol and cholesteryl esters. Apolipoproteins associated with the surface help solubilize the particle in the plasma and mediate interactions with various lipoprotein receptors, modifying proteins or metabolizing enzymes.

by the lymphatic system to the bloodstream. Very low density lipoprotein (VLDL) is the second largest lipoprotein (30-100 nm), and is synthesized and secreted by the liver via an intricate secretory pathway (Fisher and Ginsberg, 2002; Shelness and Sellers, 2001). VLDL is also rich in TG and is catabolized in the circulatory system via a complex series of reactions involving lipases and the transfer of lipids and apolipoproteins among lipoproteins (Fielding and Fielding, 1996) to produce intermediate (IDL) and low density lipoproteins (LDL; LDL is approximately 18-25 nm in diameter).

The final class is the smallest (25 nm in diameter or smaller), and these particles are known as high density lipoproteins (HDL). HDL does not carry apoB, but rather contains at least one molecule of apoAI among other apolipoproteins (Mahley *et al.*, 1984). HDL has a relatively CE-rich core. It is important for the removal of excess cholesterol from peripheral (non-hepatic) cells and its transport back to the liver for recycling or excretion as bile. The collection of cholesterol from the periphery and delivery back to the liver is known as the reverse cholesterol transport pathway. The cardioprotective nature of HDL has been shown in numerous epidemiological studies worldwide over the last 50 years. A striking inverse correlation between HDL-cholesterol and incidence of CVD has been observed (Abbott *et al.*, 1988; Castelli *et al.*, 1986; Gordon *et al.*, 1977; Husten, 1998b), strongly suggesting that a major risk factor for CVD development is low plasma HDL (Gordon *et al.*, 1989; Wilson *et al.*, 1988). Although the detailed mechanisms of how cells efflux cholesterol to HDL and how HDL protects against atherosclerosis are not fully elucidated, potential strategies to treat and prevent CVD exist in raising plasma HDL levels (Linsel-Nitschke and Tall, 2005) (Figure 1-6).

1.1.6 Initiation and progression of atherosclerosis

The initiating event for lesion formation is thought to be endothelial injury or dysfunction. This can be caused by a variety of factors, such as free radicals in cigarette smoke, hyperlipidemia, hypertension, shear stress, elevated blood sugar (diabetes mellitus), hyperhomocysteinemia, viruses or bacteria such as

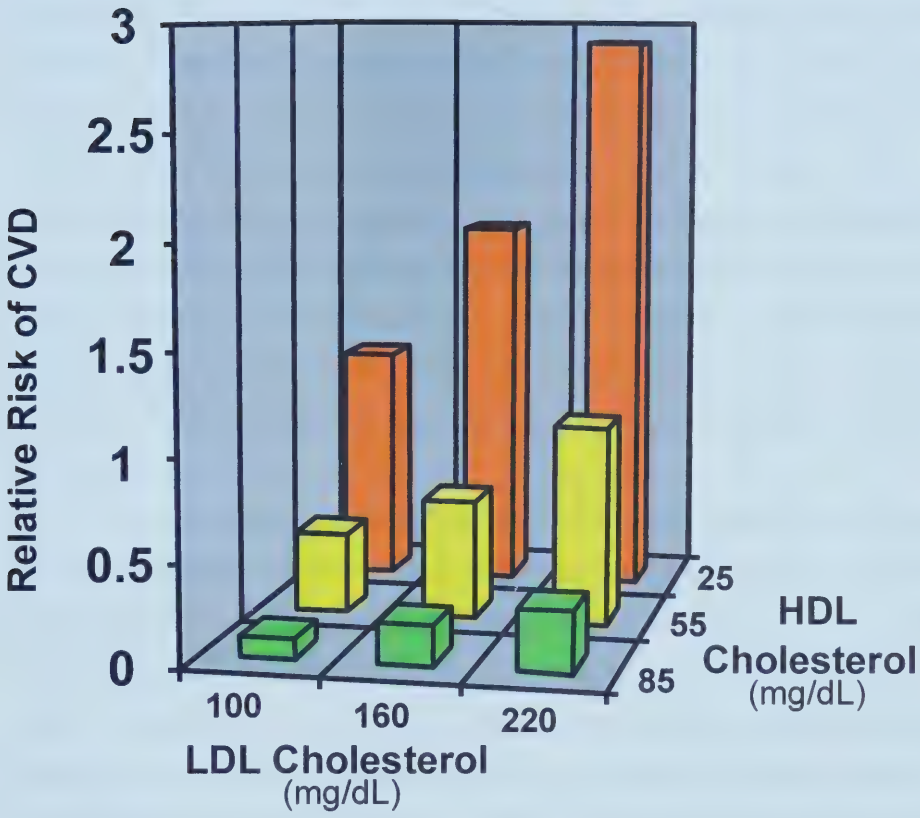

Figure 1-6 The cardioprotective nature of plasma HDL cholesterol. HDL cholesterol is an independent risk factor for the development of cardiovascular disease. Even when LDL levels are high, elevated HDL in plasma reduces the risk of CVD by a substantial amount. This figure was provided by Dr. Gordon Francis based on data from T. Gordon *et al.* (1977) *Am. J. Med.* 62:707-714.

Herpes Simplex Virus or *Chlamydia pneumoniae* as well as genetic factors that result in a predisposition (Ross, 1999). The initiating injury leads to increased endothelial permeability, as well as expression of adhesion molecules by the endothelial cells, and subsequent recruitment and infiltration of lipoproteins and leukocytes. High levels of lipids in the blood, particularly lipids carried on LDL, leads to increased opportunity for these entities to infiltrate through the endothelial layer and into the intima to contribute to pathogenesis. Once in the intima, LDL uptake and accumulation of neutral lipids in the developing atherosclerotic plaque is accomplished by macrophages that invade the arterial wall at the sites of vascular injury, then differentiate into lipid laden foam cells (Ross, 1995). Foam cells are one of the hallmarks of atherosclerotic lesions. Factors released by the leukocytes can accelerate a local immune response, leading to further lipoprotein oxidation and lipid accumulation in the macrophage foam cells.

The correlation between elevated blood lipid levels and the development of atherosclerotic lesions has been demonstrated by numerous population and epidemiological studies worldwide (Brown and Goldstein, 1983; Gordon *et al.*, 1989; Gordon *et al.*, 1977; Kannel, 1995; Kannel *et al.*, 1979; Kannel and Wilson, 1992). Similarly, a relationship between high levels of apoB (the main protein component of LDL) in plasma and the risk of cardiovascular disease has been clearly identified (Sniderman *et al.*, 1980). Reduction of CVD has been observed in men treated with lipid lowering drugs that also cause a decrease in plasma apoB (Brown *et al.*, 1990). Thus, there is a positive correlation and a mechanism for a causal relationship between the levels of apoB in plasma and atherosclerosis.

1.1.7 Plasma enzymes alter the composition of lipoproteins

As discussed above, lipoproteins play a central role in the development of CVD. Lipoprotein composition is dynamic with respect to both lipid and protein components. Cholesterol is an essential molecule for membrane structure and stability in all animal cells, and is also required for processes like steroid

hormone and bile acid synthesis. Spontaneous transfer of exchangeable apolipoproteins between particles, as well as the actions of plasma enzymes that transfer different lipid species between lipoproteins, greatly affect the overall characteristics of the particles. Phospholipid transfer protein catalyzes the transfer of phospholipids from VLDL, IDL, and LDL to HDL, a process which may provide the HDL-associated enzyme lecithin:cholesterol acyltransferase with fatty acid substrates for CE synthesis (Fielding and Fielding, 1996). Cholesteryl ester transfer protein (CETP) transfers CE from HDL to VLDL, IDL, and LDL in exchange for TG. Because it removes cholesterol from HDL, CETP is also an important determinant of plasma HDL-cholesterol levels. Whether this enzyme is pro- or anti-atherogenic remains a controversial issue, since evidence for both has been presented (Hayek *et al.*, 1995; Hirano *et al.*, 1997; Inazu *et al.*, 1990; Plump *et al.*, 1999; Sugano *et al.*, 1998; Zhong *et al.*, 1996). Small molecule inhibitors of CETP have been developed and used in clinical trials (Brousseau *et al.*, 2004; de Grooth *et al.*, 2002). These studies demonstrated an increase in plasma HDL cholesterol and a decrease in apoB containing lipoproteins induced by these agents. The results support the pro-atherogenic proposal and a therapeutic approach to lipoprotein mediated contributions to CVD.

1.1.8 Transport and uptake of lipids to peripheral (non-hepatic) cells and receptor mediated lipoprotein uptake

Cells derive lipids by both endogenous synthesis and by uptake of lipoprotein lipids or whole lipoprotein particles from the circulation. The way the liver accomplishes delivery of lipids to the periphery is by secreting VLDL, a particle with a TG-rich core. As VLDL circulates in the blood, its TG component is catabolized via several mechanisms, eventually resulting in a relatively more CE-rich LDL particle. LDL in humans is the major transporter of cholesterol synthesized by the liver to the rest of the body. Although LDL is associated with CVD, it is critical for delivery of lipids to peripheral cells. Deposition of LDL associated lipids into the arterial wall can be considered an undesired side affect of transporting lipids in this fashion. Most of the exchangeable apolipoproteins

on VLDL dissociate during the transformation into LDL (Mahley *et al.*, 1984). Apolipoprotein mediated binding of the particle to the LDL receptor (LDLR), which is displayed on the surface of cells, facilitates uptake of LDL particles. The whole particle gets internalized via endocytosis and broken down upon delivery to lysosomes (Brown and Goldstein, 1986). The lipid components are subsequently made available for various cellular requirements.

1.1.9 VLDL assembly and secretion

The major apolipoprotein component of chylomicrons, VLDL and the sole apolipoprotein of LDL is a single apoB molecule per particle (Elovson *et al.*, 1988; Fielding and Fielding, 1996). Several excellent reviews regarding the mechanism of VLDL assembly and secretion have been published (Gibbons *et al.*, 2002; Olofsson *et al.*, 1999; Shelness and Sellers, 2001). Gene targeting experiments in mice revealed that apoB is the sole apolipoprotein required for the secretion of a VLDL particle (Veniant *et al.*, 1999). ApoB is encoded by a single gene, though it occurs in two forms due to mRNA editing by a cytosine deaminase that converts codon 2153 from CAA (glutamine) to a stop codon (Chen *et al.*, 1987; Powell *et al.*, 1987). The larger form, termed apoB100, is the sole form of apoB secreted by the human liver and includes 100% of the mature protein. Human apoB100 has 4536 amino acids and a calculated molecular mass of 512 kDa (Chen *et al.*, 1986; Law *et al.*, 1986). The shorter form, apoB48, represents the amino-terminal 48% of apoB100 with 2152 amino acids and a calculated molecular mass of 264 kDa (Chan, 1992). ApoB48 is secreted by human intestine on chylomicrons for transport of dietary lipids. While apoB100 is capable of an interaction with the LDLR to mediate cellular uptake via endocytosis, apoB48 lacks the LDLR interacting domain. It is initially counter-intuitive that chylomicrons can be up to ten times larger than VLDL and carry more lipids, while at the same time they contain the shorter apoB protein. This means that the C-terminal 52% of apoB100 is not necessary for acquisition of large amounts of lipid during lipoprotein assembly. Both forms of apoB are secreted by the liver of rats and mice (Veniant *et al.*, 1999), making these

animals a more challenging model in which to investigate lipoprotein metabolism as it applies to humans.

1.2 VLDL associated lipid synthesis

1.2.1 Triacylglycerol biosynthesis

As indicated previously, TG is composed of a glycerol backbone to which three fatty acyl chains are attached via an ester linkage (Figure 1-1). The fatty acids are provided by dietary lipids or are generated *de novo*. Variations in the composition and quantity of the diet affect the rate of *de novo* fatty acid and TG synthesis to satisfy the body's demands for lipid (or energy) storage (Coleman and Lee, 2004). The source of the fatty acids for TG synthesis varies depending upon the tissue (Lehner and Kuksis, 1996). For example, the enterocyte takes up dietary fatty acids and 2-monacylglycerols (MG) and resynthesizes TG from these substrates primarily through the 2-monoacylglycerol pathway (see Section 1.2.8). Hepatocytes take up fatty acids derived from remnant lipoproteins and free fatty acids released from adipocytes. A high carbohydrate diet stimulates the synthesis of fatty acids. It is generally accepted that the synthesis of TG is controlled primarily by the amount of fatty acid available. Thus, *de novo* TG synthesis is enhanced only when fatty acid synthesis or uptake exceeds the cellular need for energy and phospholipid synthesis. In hepatocytes, as well as adipocytes, *de novo* TG synthesis is primarily achieved through the glycerol-3-phosphate pathway (see below).

1.2.2 The glycerol-3-phosphate pathway of TG biosynthesis

The glycerol-3-phosphate pathway is the main route of TG biosynthesis in non-enteric tissues (Figure 1-7). Briefly, it begins with the acylation of glycerol-3-phosphate by glycerol-3-phosphate acyltransferase (GPAT) at the *sn*-1 position, to form 1-acylglycerol-3-phosphate. Subsequently, the acylation of 1-acylglycerol-3-phosphate at the *sn*-2 position by 1-acylglycerol-3-phosphate acyltransferase (AGPAT) (also referred to as lysophosphatidic acid acyltransferase) synthesizes phosphatidic acid. The hydrolysis of phosphatidic

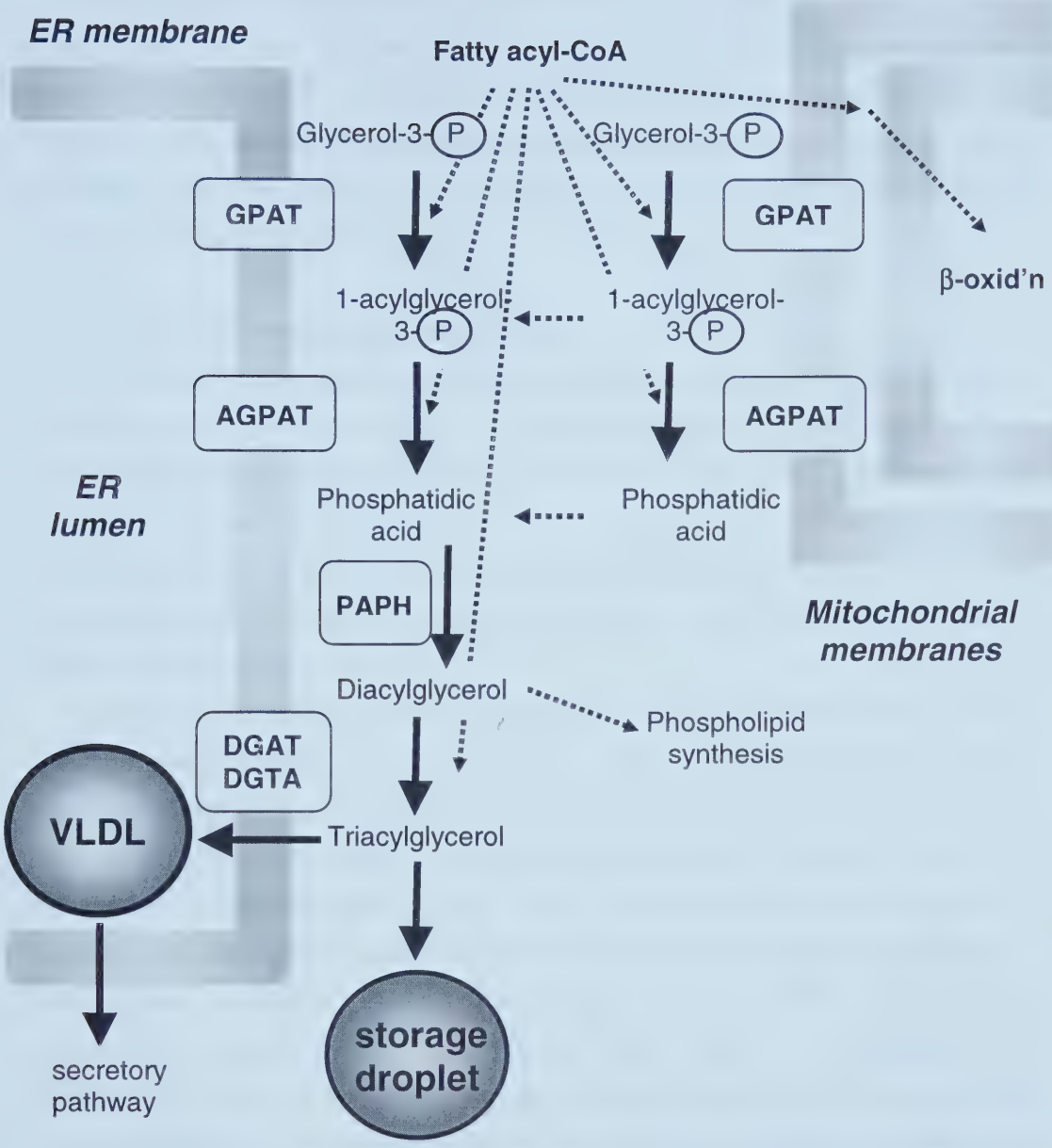

Figure 1-7 The glycerol phosphate pathway of triacylglycerol synthesis. Glycerol-3-phosphate is acylated to form 1-acyl-glycerol-3-phosphate by glycerol-3-phosphate acyltransferase (GPAT). A subsequent acylation is accomplished by 1-acyl-glycerol-3-phosphate acyltransferase (AGPAT), resulting in phosphatidic acid. Hydrolysis of the *sn*-3 phosphate through the action of phosphatidate phosphohydrolase (PAPH) produces diacylglycerol. Diacylglycerol may be converted to triacylglycerol by diacylglycerol acyltransferase (DGAT) or diacylglycerol transacylase (DGTA). Alternately, it can be diverted towards phospholipid biosynthesis. In liver, triacylglycerol is stored in droplets or secreted on VLDL.

acid by phosphatidate phosphohydrolase (PAPH) generates *sn*-1,2-diacylglycerol (DG). DG is then acylated to form TG via the action of diacylglycerol acyltransferase (DGAT). The utilization of molecular cloning and gene targeting techniques has elucidated many new insights regarding these enzymes and their regulation. The regulation of TG synthesis and its breakdown determines the quantity of TG stored within a cell.

1.2.3 Glycerol-3-phosphate acyltransferase

GPAT represents the committed step for the synthesis of all glycerolipids. Two isoforms for this enzyme exist. A mitochondrial GPAT has been cloned and demonstrated to be tightly regulated (Coleman *et al.*, 2000). A microsomal GPAT activity also exists, though a mammalian cDNA has not been cloned and its activity appears to be largely unregulated (Coleman *et al.*, 2000). The murine mitochondrial GPAT cDNA is primarily expressed in liver, adipose and muscle tissues (Paulauskis and Sul, 1988).

The nutritional and hormonal regulation of mitochondrial GPAT is well characterized in mice, and occurs primarily at the transcriptional level. Post-translational reduction of mitochondria GPAT activity has been reported to occur upon stimulation of adenosine monophosphate-activated kinase, a sensor of cellular fuel deprivation (Muoio *et al.*, 1999). GPAT activity has strong links to obesity. For example, Zucker rats are a commonly used model for obesity as well as insulin resistance. These animals exhibit enhanced GPAT activity in both hepatic and adipose tissues (Jamdar and Cao, 1995). The regulation of mitochondrial GPAT is consistent with a function in reducing TG synthesis and increasing oxidation when nutrients are limiting, and conversely to direct fatty acids toward TG synthesis and away from oxidation when energy is in excess (Ericsson *et al.*, 1997). The localization of this GPAT activity on the cytosolic surface of mitochondria also suggests a role in preventing fatty acids (more accurately fatty acyl-CoAs) from entering this organelle and undergoing β-oxidation under conditions of metabolic excess by converting it to 1-acylglycerol-3-phosphate and thus directing the fatty acid toward TG synthesis (Figure 1-7).

1.2.4 Acylglycerol-3-phosphate acyltransferase

This enzyme is also referred to as lysophosphatidic acid acyltransferase (Coleman *et al.*, 2000). Several AGPAT cDNA sequences have been identified. Human and mouse cDNAs of 2kb and 1.8 kb respectively, that encode proteins with AGPAT activity, were identified and designated AGPAT-1 (Kume and Shimizu, 1997; Stamps *et al.*, 1997). 3T3-L1 cells are a fibroblastic pre-adipocyte cell line that can be differentiated into cells with an adipocyte phenotype. AGPAT-1 overexpression in 3T3-L1 adipocytes inhibited fatty acid release while promoting its uptake and TG storage (Ruan and Pownall, 2001). Expression of AGPAT-1 mRNA has been observed in all tissues examined, with highest expression in skeletal muscle (Kume and Shimizu, 1997; Stamps *et al.*, 1997). AGPAT-2 mRNA is expressed at highest levels in the liver, heart and adipose tissues, and much less in other tissues (Eberhardt *et al.*, 1997; Eberhardt *et al.*, 1999). The protein encoded by the AGPAT-2 cDNA shares 48% homology with AGPAT-1 and is expressed at two-fold higher levels than AGPAT-1 in the adipose tissue, similar levels in liver and AGPAT-1 is expressed at 1.8-fold greater levels than AGPAT-2 in skeletal muscle (Agarwal *et al.*, 2002). AGPAT-1 and 2 were localized to the endoplasmic reticulum (ER) membrane (Aguado and Campbell, 1998; Leung, 2001). Several individuals with congenital generalized lipodystrophy were discovered to have a variety of different mutations in the AGPAT-2 gene that led to the production of a dysfunctional protein (Agarwal *et al.*, 2002). These observations suggest that AGPAT-2 is necessary for TG synthesis in adipocytes. Three additional AGPAT cDNAs have been designated AGPAT-3,4,5 respectively. The corresponding mRNAs are barely detectable in liver, adipose and skeletal muscle and do not yet have a clearly identified function (Agarwal *et al.*, 2002).

1.2.5 Phosphatidate phosphohydrolase

Two forms of PAPH activity exist, PAPH-1 and PAPH-2 (also called lipid phosphate phosphohydrolase). Several cDNAs for the PAPH-2 family or lipid

phosphate phosphohydrolases have been cloned. These enzymes appear to play a role in signal transduction pathways by generating the lipid second messenger, DG (Brindley and Waggoner, 1998). PAPH-1 activity exists in both cytosol and microsomal fractions. It has been proposed that long-chain fatty acids stimulate the translocation of PAPH-1 from the cytosol to the ER membrane where PA is hydrolyzed to DG (Gomez-Munoz *et al.*, 1992; Martin *et al.*, 1987). A cDNA encoding an enzyme with PAPH-1 activity has not been cloned, but this activity is considered the rate limiting step for *de novo* TG synthesis. There is a strong correlation between the activity of membrane associated PAPH-1 and the rate of TG synthesis (Tijburg *et al.*, 1989). PAPH-1 activity is subject to dietary and hormone-induced changes (Tijburg *et al.*, 1989). PAPH-1 activity was increased 2-3 fold by glucagon, cyclic adenosine monophosphate (cAMP) and dexamethasone in primary rat hepatocytes (Pittner *et al.*, 1986; Pittner *et al.*, 1985a; Pittner *et al.*, 1985b). It was hypothesized that enhanced PAPH-1 activity enables the liver to increase its ability to synthesize TG during the period of maximum feeding.

1.2.6 Diacylglycerol acyltransferase

DGAT catalyzes the final step in TG synthesis. Two DGAT cDNAs have been cloned. DGAT-1 is ubiquitously expressed in human and mouse tissues, with the highest levels of expression in the small intestine, colon, testis, thymus, heart and skeletal muscle (Cases *et al.*, 1998). The mammalian DGAT-2 cDNA is unrelated to the mammalian DGAT-1 cDNA and was cloned based on its homology with a fungal DGAT cDNA (Cases *et al.*, 2001). Both proteins have been localized to microsomal membranes. DGAT-2 is highly expressed in liver and adipose, which correlates with a high capacity to synthesize TG in these tissues. Analysis of the predicted amino acid sequence from the DGAT-2 cDNA suggests the presence of a single transmembrane segment and a domain with homology to the glycerol binding domain of GPAT and AGPAT. The glycerol binding domain of DGAT-2 is predicted to be within the ER lumen where the synthesis of TG would be ideally linked to the site of VLDL assembly. There is

experimental evidence for the existence of overt (cytosolic) and latent (lumenal) DGAT activities in microsomes (Owen *et al.*, 1997). Hence, different acyltransferases (or transacylases), acting in different subcellular compartments could be involved in the synthesis of TG, and the extent of their respective activities could channel TG preferentially for storage within a cytosolic storage pool or towards the secretory pathway via assembly into lipoproteins. Whether the DGAT-1 or -2 enzyme is responsible for directing TG towards VLDL versus storage has been investigated.

Recently, adenovirus constructs were used to overexpress DGAT-1 or DGAT-2 in mice (Yamazaki *et al.*, 2005). Intriguingly their data showed that DGAT-1 overexpression led to increased VLDL, while DGAT-2 overexpression led to TG accumulation in the liver. This supports the hypothesis that DGAT-1 activity directs TG synthesis to the lumen of the ER where it is then available for VLDL assembly, while DGAT-2 activity directs TG to cytoplasmic storage pools.

The precise contribution of DGAT-1 to TG synthesis was also examined in mice that had the *Dgat*-1 gene disrupted. The targeted deletion of the *Dgat*-1 gene failed to eliminate the synthesis of TG in many tissues and the production of plasma TG was normal (Chen *et al.*, 2002a; Smith *et al.*, 2000). *Dgat1*$^{-/-}$ mice had dry fur that did not repel water, and female mice had a defect in their ability to lactate (Smith *et al.*, 2000). In addition, DGAT-1 null mice had a 50% reduction in fat pad content and were resistant to weight gain when fed a high fat diet that was associated with a 15% increase in daily total energy expenditure. DGAT-1 null mice had increased sensitivity to insulin and leptin. When *Dgat1*$^{-/-}$ mice were crossed into the background of the obese leptin deficient *ob/ob* mouse, DGAT-1 deficiency did not affect energy and glucose metabolism (Chen *et al.*, 2002a). In addition, DGAT-2 expression was increased in *ob/ob* mice, suggesting that the leptin pathway directly down regulates DGAT-2 expression. In the absence of leptin, DGAT-2 sufficiently compensated for the loss of DGAT-1 mediated TG synthesis (Chen *et al.*, 2002a; Chen *et al.*, 2002b). Further, others have observed a 3-fold increase in overt and latent DGAT activities in liver microsomes from obese *ob/ob* mice relative to lean controls (Waterman and

Zammit, 2002). DGAT-1 was not necessary for intestinal TG absorption and chylomicron synthesis, although a high fat diet caused accumulation of cytosolic TG within the enterocytes of *Dgat1*$^{-/-}$ mice (Buhman *et al.*, 2002). Together, these results suggest that DGAT-2 (or possibly diacylglycerol transacylase – see below) activities sufficiently compensated for the targeted deletion of the DGAT-1 gene in many tissues.

Disruption of the *Dgat*-2 gene resulted in lethality shortly after birth (Stone *et al.*, 2004), revealing a developmental requirement of this gene product that is not compensated by DGAT-1. Surprising insights into a role of DGAT-2 in provision of lipids involved in regulating the permeability of the skin were also revealed. The skin defects in these mice were much more profound than those in DGAT-1 null mice. The phenotypes demonstrate that DGAT-1 and -2 have different roles in TG metabolism. Tissue specific disruption of *Dgat*-2 may contribute to a better understanding of how this enzyme participates in various cellular processes.

1.2.7 Diacylglycerol transacylase

As indicated in Figure 1-7, fatty acyl-coenzyme A (acyl-CoA) plays a central role in TG biosynthesis. Acyl-CoA independent mechanisms for TG formation are also present in animal tissues. The isolation of a diacylglycerol transacylase (DGTA) from enteric microsomes demonstrated that the transacylation between two 1,2-DG molecules to form TG and MG occurs (Lehner and Kuksis, 1993b). The relative contribution of this pathway to TG synthesis is unknown because enzymes of the monoacylglycerol and phosphatidic acid pathways exist in the same subcellular compartment and must be dissected apart to discern these details. DGTA activity is low when compared to total DGAT activity (Lehner and Kuksis, 1992; Lehner and Kuksis, 1993b), however, the presence of DGTA may be important for the synthesis of TG within the ER lumen, since transport of acyl-CoA across the ER membrane would not be required for this route of TG synthesis (Abo-Hashema *et al.*, 1999).

1.2.8 Monoacylglycerol pathway of TG biosynthesis

Within the intestinal lumen, lipases hydrolyze TG to free fatty acids and 2-MG. MG is taken up by enterocytes and undergoes resynthesis back to TG. This process is initially catalyzed by monoacylglycerol acyltransferase (MGAT) to synthesize DG, which then serves as a substrate for DGAT or DGTA. Most of the TG generated by the intestine is secreted as chylomicron particles, which are described in Section 1.1.5.

1.2.9 Monoacylglycerol acyltransferase

MGAT activity in the small intestine is primarily responsible for the acylation of dietary MGs. In liver, MGAT activity is higher in diabetic animals, but low in obese Zucker rats (Jamdar and Cao, 1995; Mostafa *et al.*, 1993). Significant levels of MGAT activity are detected in white adipose tissue and kidney (Coleman and Haynes, 1984; Jamdar and Cao, 1995). The different chromatographic and inhibition profiles of the rat intestinal and hepatic MGAT activities suggested that MGAT activity represents tissue specific isoenzymes (Manganaro and Kuksis, 1985a; Manganaro and Kuksis, 1985b). A mouse cDNA was identified as encoding a protein that possessed MGAT activity when expressed in insect cells (Yen *et al.*, 2002). This gene, designated as MGAT-1, is expressed in stomach, kidney, liver and adipose tissue in mice. Interestingly, MGAT-1 was not expressed in the small intestine, again suggesting the existence of an additional MGAT gene. Following this, MGAT-2 and -3 have been identified, and their respective cDNAs have been cloned (Cao *et al.*, 2003; Cheng *et al.*, 2003). MGAT-2 shows highest expression in the small intestine, with lower amounts of the mRNA detectable in kidney, adipose and stomach. This enzyme was described as also possessing a weak DGAT activity in transfected cells. MGAT-3 expression appears to be exclusive to the small intestine and is most abundant in the ileum. Both MGAT-2 and -3 are thus implicated in the process of dietary fat absorption. The predominance of the MGAT pathway of TG synthesis in the intestine is coincident with the expression of MGAT-2 and -3 in this tissue.

1.2.10 Subcellular distribution of fatty acyl-CoA for lipid synthesis

The subcellular localization of activated fatty acid, that is fatty acyl-CoA, at the ER membrane for utilization by various acyltransferases including DGAT and MGAT is presently unclear. One possibility is that microsomal acyltransferases (which are typically transmembrane proteins) utilize acyl-CoA strictly from the cytosolic pool. A second possibility is the transfer of fatty acids across the ER membrane as acylcarnitine derivatives. Microsomal carnitine acyltransferases have been demonstrated to exist (Broadway and Saggerson, 1995b; Murthy and Pande, 1994). Tolbutamide is a sulfonylurea drug that inhibits mitochondrial carnitine acyltransferase activity (Cook, 1987). Since tolbutamide also inhibits microsomal carnitine acyltransferase and suppresses VLDL secretion by hepatocytes, it seems reasonable to hypothesize that the microsomal carnitine acyltransferase system is an important component for the delivery of fatty acids to the ER lumen (Broadway and Saggerson, 1995a; Wiggins and Gibbons, 1992). However, more recent studies suggest that the observed microsomal carnitine acyltransferase activities may have been a result of contamination of microsomes with mitochondria (Broadway *et al.*, 2003; Broadway *et al.*, 2001).

1.2.11 Phosphatidylcholine biosynthesis

The primary phospholipid found on lipoproteins is phosphatidylcholine (PC) (Davis and Vance, 1996). Two biochemical pathways for PC synthesis exist. One pathway is essentially liver specific and involves the conversion of phosphatidylethanolamine to PC via three consecutive methylation reactions that are all catalyzed by the enzyme phosphatidylethanolamine N-methyltransferase (PEMT) (Vance *et al.*, 1997). *S*-adenosylmethionine is utilized by this enzyme as the donor of the methyl group to produce *S*-adenosylhomocysteine, which is subsequently converted to homocysteine. Plasma homocysteine levels are an independent risk factor for the development of CVD (Refsum *et al.*, 1998). Disruption of the *Pemt* gene resulted in a 50% reduction in plasma homocysteine in mice (Noga *et al.*, 2003), demonstrating that the PEMT reaction has a very

significantly impact on plasma levels of this molecule, and is important with respect to CVD development beyond VLDL production. The PEMT null mice also rapidly develop liver failure on a choline deficient diet compared to *wild type* mice (Walkey *et al.*, 1998). The mechanism for this is related to a sensitivity towards the loss of PC in bile (Vance and Vance, 2005).

The predominant pathway for PC synthesis is found in all nucleated cells (Kent, 1997). The process begins with the uptake of dietary choline, as choline is not manufactured in animal cells other than via methylation of phosphatidylethanolamine to PC followed by hydrolysis of the choline group (Vance, 1996). Following uptake, choline is rapidly phosphorylated to phosphocholine by choline kinase (Uchida and Yamashita, 1992). Phosphocholine is combined with cytidine triphosphate (rather than adenosine triphosphate encountered in many biological reactions) in a rate limiting reaction catalyzed by cytidine triphosphate: phosphocholine cytidylyltransferase (CCT) to form cytidine diphosphocholine (CDP-choline) (Vance, 1996). There are two mammalian genes that encode isoforms of CCT, and the two genes exhibit tissue specific expression. One gene encodes CCTα, which is expressed in most tissues (Lykidis *et al.*, 1999), with high expression in testis, lung, liver and ovary. Subcellularly, CCTα is found most abundantly in the nucleus (Wang *et al.*, 1993), with lower amounts associated with the ER and the cytosol (Lykidis *et al.*, 1999). The second gene encodes CCTβ, which is most predominant in the brain (Karim *et al.*, 2003), and is localized to the ER. Alternate splicing of the CCTβ gene result in at least two mRNAs termed CCTβ2 and CCTβ3. Current biochemical data have not distinguished a unique, nonredundant role for the CCTβ isoforms in cell or tissue function, although disruption of CCTβ2 produced gonadal dysfunction in both male and female mice (Jackowski *et al.*, 2004). No overt defects were found in the brain. An additional splice variation of the CCTβ gene gives rise to a CCTβ1 transcript found in the expressed sequence tag database (Karim *et al.*, 2003), however, the protein has not been detected in either mouse or human tissues. Therefore, the existence of this protein remains speculative.

In a final step towards this phospholipid synthesis, CDP-choline is combined with 1,2-diacylglycerol to form PC in a reaction catalyzed by CDP-choline: 1,2-diacylglycerol cholinephosphotransferase. This enzyme and PEMT are localized to the ER where the majority of all phospholipid synthesis occurs. It is currently unclear which pathway is of primary importance with respect to VLDL assembly, as evidence for both has been presented (Jacobs *et al.*, 2004; Noga and Vance, 2003; Noga *et al.*, 2002).

1.2.12 Summary of cholesterol biosynthesis

Cholesterol and CE are relatively minor, but important components of VLDL (Davis and Vance, 1996). Beyond lipoproteins, cholesterol is used for membrane biogenesis, cell growth, steroid hormones and bile acids (reviewed in (Liscum and Munn, 1999)). The cholesterol biosynthetic pathway is complex and involves numerous enzymes, which are localized to the cytosol, ER and peroxisomes (Olivier and Krisans, 2000). The ultimate synthesis of this molecule occurs in the ER. The entire cholesterol biosynthetic route will not be reviewed here. The rate limiting step in cholesterol biosynthesis occurs early in the pathway, and is catalyzed by 3-hydroxy-3-methylglutarly-CoA reductase. This enzyme's activity is regulated by cellular cholesterol levels. Chemical inhibition of this enzyme by the statin family of drugs has been used therapeutically to reduce plasma cholesterol levels (Ganesh *et al.*, 2003). Owing to their ability to lower plasma LDL cholesterol, the use of statins has shown a marked reduction in coronary events, especially in people with hypercholesterolemia.

Because cholesterol in membranes affects their fluidity, cholesterol is esterified for storage in lipid droplets. As described in Section 1.1.3, excess accumulation of cholesterol and CE in atherosclerotic lesions is a hallmark of the disorder. Esterification is mediated by acyl-CoA:cholesterol acyltransferase (ACAT) (Farese, 1998). ACAT is thought to play an important role in membrane biology by maintaining the free sterol content of membranes within ranges optimal for proper cell function. Two isoforms of ACAT are encoded by separate genes (Joyce *et al.*, 1999). ACAT-1 is found ubiquitously throughout the body,

while ACAT-2 is expressed in liver and intestine. Both isoforms are localized to the ER membrane.

1.2.13 Regulation of lipid synthesis by SREBP

Biosyntheses of cholesterol, glycerolipids and fatty acids are regulated by isoforms of a transcription factor known as sterol regulatory element-binding protein (SREBP) (Horton *et al.*, 2002; Shimano, 2001). SREBPs bind sterol regulatory elements (SREs) found in the proximal promoters of target genes and upregulate their expression.

SREBPs are synthesized in the ER and are initially trans-membrane proteins. In the ER, SREBPs are bound to SREBP cleavage-activating protein (SCAP) (Brown and Goldstein, 1997; Horton *et al.*, 2002). SCAP is also a membrane protein that is required for movement of SREBPs from the ER to the Golgi. In the Golgi, a two-step proteolytic cleavage results in release of a soluble, N-terminal, transcriptionally active fragment of SREBP that can enter the nucleus and activate target genes involved in sterol and fatty acid synthesis (Brown and Goldstein, 1999). SCAP/SREBP trafficking is regulated by cholesterol via a "sterol-sensing domain" present in SCAP (Nohturfft *et al.*, 1999).

To date, three major SREBPs have been identified, and they are designated SREBP-1a, -1c and 2. SREBP-1 regulates fatty acid and TG synthesis, whereas SREBP-2 regulates cholesterol synthesis (Horton *et al.*, 2002). SREBP-1a and -1c are generated from a single gene through the use of alternate promoters, and SREBP-2 is a product of a separate gene (McPherson and Gauthier, 2004). Hence SREBP-1a and -1c are identical except for their N-terminal transactivation domains. SREBP-1a has a longer N-terminal transactivating domain and binds more strongly to SREs than SREBP-1c (Shimano *et al.*, 1997). As such, SREBP-1a is a potent activator of all SREBP-responsive genes, including those that mediate the synthesis of cholesterol, fatty acids, and triglycerides.

Many SREBP-1c responsive genes have major influence on TG synthesis or VLDL assembly. SREBP-1c responsive genes include fatty acid synthase

(Magana and Osborne, 1996), acetyl-CoA carboxylase (Magana *et al.*, 1997), long-chain fatty acyl-CoA elongase (Moon *et al.*, 2001) and stearoyl-CoA desaturase-1 (section 1.3.2) (Tabor *et al.*, 1998). SREs are also present in the promoters of genes encoding GPAT (section 1.2.3) (Ericsson *et al.*, 1997) and the rate-controlling step in phosphatidylcholine synthesis, CCT (section 1.2.11) (Lagace *et al.*, 2000). The identification of SREBPs and characterization of their mechanism of action has been a critical advance in the understanding of lipid homeostasis.

1.3 VLDL assembly and secretion

1.3.1 Provision of lipid for VLDL assembly

The liver has the ability to store neutral lipids or secrete them into the circulation as VLDL particles, thereby regulating storage and secretion with the energy needs of the body (Olofsson *et al.*, 1999; Shelness and Sellers, 2001). Exogenous fatty acids taken up by hepatocytes are not directly utilized for secretion as VLDL associated TG, but enter an intracellular storage pool of TG (Diraison and Beylot, 1998; Gibbons *et al.*, 1992). In the post-absorptive state, stored TG is released by the liver as VLDL (Diraison and Beylot, 1998). On average, a normal adult human liver stores about 5 μmol TG/g of liver weight, though the liver has the capacity to store much larger quantities of TG (Gibbons *et al.*, 2000).

A plethora of studies have been performed in hepatocytes to investigate VLDL assembly and secretion. These include the hepatoma cell lines McArdle RH7777 and HepG2 as well as primary hepatocytes isolated from rats, hamsters and mice. ApoB, the main protein component of VLDL, is translated on ribosomes associated with the rough ER. The transcription and translation of apoB is continuous so that newly synthesized apoB is always available for assembly with lipids for secretion (Pullinger *et al.*, 1989). Lipid availability determines the percentage of apoB that is assembled into secretion-competent lipoprotein particles versus that which is misfolded and targeted for degradation (Borchardt and Davis, 1987; Fisher and Ginsberg, 2002; Pullinger *et al.*, 1989;

White *et al.*, 1992). The relative importance of each core lipid in the assembly of VLDL is a fundamental issue that has been explored using a variety of methods. There is general agreement that stimulation of hepatic TG synthesis will also augment the secretion of TG and apoB (Boren *et al.*, 1993; Fisher and Ginsberg, 2002; Yao and McLeod, 1994). In contrast, there is debate over whether or not CE biosynthesis alters VLDL and apoB secretion. Mice virtually devoid of hepatic ACAT activity, the enzyme catalyzing CE formation, still synthesize and secrete apoB containing lipoproteins, although the particles were of smaller size than those produced from *wild type* mice (Buhman *et al.*, 2000). Counter to these studies, however, the overexpression of ACAT in McArdle RH7777 cells resulted in increased synthesis, cellular accumulation and secretion of CE (Liang *et al.*, 2004). Decreased intracellular degradation and increased secretion of apoB were also observed. Overexpression of DGAT-1 in this cell line had similar effects in that increased TG levels and apoB secretion were encountered, reflecting the critical importance of neutral lipid availability in VLDL production.

It is generally accepted that the assembly of VLDL particles takes place in two or more steps (Fisher and Ginsberg, 2002; Innerarity *et al.*, 1996). Studies using McArdle RH7777 cells and primary rat hepatocytes demonstrated that the newly translated apoB is not integrated into microsomal membranes, but is initially associated with the lumenal side of the ER membrane in a peripheral fashion (Hebbachi and Gibbons, 2001; Rustaeus *et al.*, 1998). In the first step of VLDL assembly, apoB is partially lipidated with small quantities of TG, phospholipid and cholesterol forming a dense primordial particle. Since apoB is a large hydrophobic protein, chaperone assisted folding of apoB appears to be necessary during its translation and translocation into the ER lumen (Chen *et al.*, 1998; Linnik and Herscovitz, 1998). The subsequent addition of the bulk of the neutral lipid to the small dense apoB containing entity represents the second step and results in a very low density apoB containing particle that may be secreted from the hepatocyte and into the circulatory system.

There is currently debate in the literature about the location and mode of assembly of VLDL particles. Several studies demonstrated that both steps in

VLDL assembly are complete before the particle enters the Golgi apparatus in primary hepatocytes and in McArdle RH7777 cells (Cartwright and Higgins, 1995; Glaumann *et al.*, 1975; Yamaguchi *et al.*, 2003). Alternately, completion of VLDL assembly may occur post-ER or in the Golgi compartment (Gusarova *et al.*, 2003; Tran *et al.*, 2002).

1.3.2 Regulation of VLDL secretion

In hepatoma cells, active synthesis of lipids drives VLDL secretion. Oleic acid promotes apoB secretion by hepatoma cells through increased TG synthesis and lipid availability (Boren *et al.*, 1994; Sakata *et al.*, 1993). Triacsin D blocked oleic acid stimulated apoB secretion through the inhibition TG synthesis without affecting CE synthesis (Wu *et al.*, 1994). It has been observed that CE synthesis has a regulatory role in VLDL synthesis (Spady *et al.*, 2000; Zhang *et al.*, 1999). However targeted deletion of the ACAT genes in mice failed to demonstrate that CE synthesis was essential for lipoprotein production (Buhman *et al.*, 2000; Meiner *et al.*, 1996). PC synthesis, a quantitatively small component of VLDL, is a potential regulator of VLDL assembly and secretion (Jacobs *et al.*, 2004; Noga *et al.*, 2002; Yao and Vance, 1988).

Stearoyl-CoA desaturase (SCD) catalyzes the synthesis of monounsaturated fatty acids from saturated fatty acids. SCD-1 deficient mice have impaired TG and CE synthesis (Miyazaki *et al.*, 2000). Furthermore, these mice secrete lower levels of TG on VLDL (Miyazaki *et al.*, 2000; Miyazaki *et al.*, 2001). SCD-1 expression is tightly regulated by insulin and carbohydrates (Shimomura *et al.*, 1998). High carbohydrate diets enhance the synthesis and secretion of TG by the liver and the hepatic secretion of TG on VLDL by SCD-1 deficient mice do not respond to a high carbohydrate diet, suggesting that SCD-1 can regulate VLDL secretion (Boogaerts *et al.*, 1984; Shimomura *et al.*, 1998).

1.3.3 Origin of TG for VLDL assembly and the role of microsomal triglyceride transfer protein

A lingering debate addresses the location of the storage pool of TG that is utilized for lipidation of primordial apoB containing particles (the 2nd step of apoB lipidation). Fatty acids can be synthesized or taken up by hepatocytes, but enter an intracellular storage pool as TG and are not secreted directly (Wiggins and Gibbons, 1992). The bulk of hepatic TG is stored in cytoplasmic lipid droplets. It remains unclear how TG from the cytosol-facing ER-associated TG droplet is recruited to the lumen of the ER for VLDL assembly. A non-apoB associated neutral lipid droplet has been observed by electron microscopy within the lumen of the smooth ER, and hypothesized to be the source of the bulk of the lipid for the primordial VLDL particle (Alexander *et al.*, 1976). Non-apoB associated neutral lipid droplets in the ER lumen of hepatocytes have subsequently been demonstrated in several reports (Kulinski *et al.*, 2002; Wang *et al.*, 1999b). Since TG cannot diffuse across the ER bilayer *en bloc,* the lumenal lipid droplet is presumably derived from both *de novo* synthesized TG and from cytoplasmic TG stores in a process that involves microsomal triglyceride transfer protein (MTP). MTP is composed of a 97 kDa subunit that is complexed with the ER folding chaperone protein disulfide isomerase (PDI) (Berriot-Varoqueaux *et al.*, 2000; Gordon and Jamil, 2000). MTP has the ability to transfer lipid between liposomes. This activity appears to be essential for transferring the bulk of triglycerides into the lumen of the ER for VLDL assembly (Raabe *et al.*, 1999). MTP dependent formation of apoB free TG-rich droplets within the ER lumen may fuse with the primordial apoB containing particle (Hamilton *et al.*, 1998). While the formation of the lipid droplet within the ER lumen is thought to be dependent upon MTP, the bulk lipidation of the apoB particle may be MTP independent (Gordon *et al.*, 1996; Raabe *et al.*, 1999; Rustaeus *et al.*, 1998; Wang *et al.*, 1999b). Inhibition of MTP caused apoB to be co-translationally degraded at an early stage in lipoprotein assembly (Zhou *et al.*, 1998).

MTP has been identified as the defective protein in abetalipoproteinemia (Gregg and Wetterau, 1994; Wetterau *et al.*, 1992). Abetalipoproteinemia is a

rare disease associated with the absence of apoB containing lipoproteins in plasma and malabsorption of fat soluble vitamins causing severe spinocerebellar and retinal degeneration. In mice, homozygous disruption of the *Mtp* gene results in embryonic lethality (Raabe *et al.*, 1998). Liver specific gene disruption of *Mtp* resulted in striking reductions in plasma apoB, VLDL triglycerides and large reductions in both VLDL/LDL and HDL associated cholesterol (Raabe *et al.*, 1999). Surprisingly, loss of MTP expression was associated with a nearly complete absence of apoB100 in hepatic microsomes. Levels of microsomal lumenal chaperone proteins [e.g., PDI, glucose-regulated protein 78 (GRP78), and GRP94] and cytosolic heat shock proteins (e.g., HSP60, HSC, HSP70, and HSP90) were unaffected by MTP inhibition. MTP inhibitors have been used as therapeutic agents in clinical trials for hypercholesterolemia in humans (reviewed in (Sudhop and von Bergmann, 2002)). Although very impressive results were achieved with respect to plasma lipids, safety concerns arose regarding absorption of fat soluble vitamins as well as steatosis (fatty degradation) of the liver.

1.3.4 Stored TG undergoes lipolysis and re-esterification prior to VLDL assembly

As described above, substantial progress has been made in our understanding of the pathways of TG biosynthesis, storage and recruitment for further metabolism. Several studies from various laboratories have demonstrated that only a minor fraction of TG in VLDL originates from *de novo* synthesis, while the majority (60-70%) is derived from preformed TG storage pools (reviewed by (Gibbons *et al.*, 2000)). Further, several groups, using different experimental approaches, have quantitatively determined that stored TG within hepatocytes undergoes a cycle of lipolysis followed by re-esterification prior to secretion on VLDL particles. A proportion of the TG is secreted as VLDL, though the majority of the re-esterified TG gets returned to storage depots.

Wiggins and Gibbons (Wiggins and Gibbons, 1992) pre-labeled the stored TG of cultured primary rat hepatocytes with [^{3}H] oleate and [^{14}C] glycerol and observed the distribution of the labeled TG over a subsequent 24 h chase period

in the absence of exogenously supplied fatty acids. These authors contend that any changes in the specific radioactivity of the two isotopes in TG requires uncoupling of [^{3}H] oleate and [^{14}C] glycerol, which represents lipolysis followed by esterification. If the extent of decline in [^{14}C] and [^{3}H] specific radioactivity is of the same magnitude, then the net lipolysis and esterification is zero, *de novo* synthesized TG can be supplied directly for secretion and the decline in the specific activity of cellular and secreted TG would reflect the extent of *de novo* TG synthesis. Two significant observations were made by this study: 1) A 70% decline of the VLDL-TG [^{14}C] label indicated that the original TG-glycerol pool was diluted with unlabeled glycerol and 2) The quantity of hydrolyzed TG that returned to the intracellular storage pool amounted to 1 pool per day, which was estimated to be 2-3 times greater than required to maintain TG secretion. Because the original TG-glycerol pool was diluted with unlabeled glycerol, lipolysis and re-esterification seemed to be essential for VLDL-TG assembly. The majority of TG that undergoes lipolysis and re-esterification is returned to storage pools.

Lankester *et al.* (Lankester *et al.*, 1998) used a dual radio-labeling technique to differentiate between the incorporation of acyl chains into TG that were derived from exogenous and endogenous fatty acids. The stored TG was pre-labeled with [^{3}H] oleate and then cultured a further 3 h with [^{14}C] oleate. The authors observed exogenous [^{14}C] fatty acids contributed only approximately 17% of total acyl chains secreted as TG, indicating that the majority of the acyl chains were derived from the pre-labeled TG stores. Furthermore, the authors demonstrated that the TG was not hydrolyzed completely to fatty acid and glycerol. Since the [^{3}H] fatty acids derived from radio labeled TG was not available to the same extent as exogenous [^{14}C] fatty acids for oxidation, [^{3}H] labeled TG undergoes incomplete lipolysis to DG and is available for resynthesis as secreted TG.

Yang *et al.* (Yang *et al.*, 1995) used chiral and reverse phase high pressure liquid chromatography with mass spectrometry to reveal similarities in positional distribution and molecular association in the 1,2-DG acyl chains of

secreted TG on VLDL and stored hepatic TG. However, the 2,3-DG acyl chains of secreted TG on VLDL were different from the stored hepatic TG. These authors calculated that 60% of secreted TG was derived via lipolysis to DG followed by re-esterification and 40% of secreted TG could have been derived from *de novo* TG synthesis. These authors added further evidence through the analyses of secreted TG following labeling of stored TG by [^{3}H] fatty acids or glycerol. The authors deduced that 30-40% of the glycerol and fatty acids in TG on VLDL are not direct products of TG stored within the liver (Yang *et al.*, 1996). Taken together, the data is consistent with a proportion of the TG stored within the liver undergoing lipolysis to DG/MG, which is re-esterified to TG that is available for secretion on VLDL or returned to storage pools in a futile cycle.

1.3.5 Regulation of hepatic TG lipolysis and re-esterification

Since it has been demonstrated that intracellular TG lipolysis and re-esterification was necessary for the efficient recruitment of stored TG for VLDL assembly, then altering the rate of hepatic TG lipolysis and re-esterification could control the rate of VLDL secretion. Glucose increased hepatic TG secretion and increased the dilution of the glycerol label in pre-labeled TG, consistent with increased lipolysis and re-esterification (Boogaerts *et al.*, 1984; Brown *et al.*, 1999; Durrington *et al.*, 1982). In addition, it was found that glucose phosphorylation was a necessary event for increased lipolysis and re-esterification since mannoheptulose, an inhibitor of glucose phosphorylation, abolished the stimulatory effect of glucose (Brown *et al.*, 1999).

1.3.6 Enzymes involved in providing TG for VLDL should localize to the ER

Since apoB containing lipoproteins are at least partly assembled within the ER lumen, it is likely that the synthesis of TG for VLDL assembly must be directed towards that compartment. The molecular mechanism and intracellular location of the enzymes responsible for the lipolysis and resynthesis of TG for VLDL secretion remain obscure, but requires ER-localized DGAT and possibly MGAT. The TG lipolysis may take place at sites where the ER membrane is in

contact with cytosolic lipid droplets (Blanchette-Mackie *et al.*, 1995; Horton *et al.*, 1999). At these contact points, lipolytic products would have increased solubility within the ER membrane and would encounter the TG synthetic enzymes located within the ER membrane as opposed to being directed towards the oxidative pathway in the mitochondria (Gibbons *et al.*, 2000). This ER localization would promote resynthesis of TG at or in proximity to the site of VLDL assembly. TG that is not assembled onto apoB may be returned to storage pools. There has been substantial progress towards identifying and characterizing the lipase responsible for mobilizing stored TG for VLDL assembly. It has been established that this lipolysis is not catalyzed by lysosomal (acidic) lipase (Wiggins and Gibbons, 1992). Additionally, the well described cytosolic hormone-sensitive lipase (HSL) (Strålfors *et al.*, 1987) is not found in appreciable quantities in the liver. Importantly, expression of HSL in HepG2 cells directed fatty acids into the oxidative pathway as opposed to the secretory pathway (Pease *et al.*, 1999), supporting the concept that the subcellular localization of any TG lipase is crucial in directing the fate of the products of lipolysis.

1.4 Triacylglycerol Hydrolase (TGH)

1.4.1 Purification of TGH and cloning of cDNAs

It was an important advance when TGH was purified from porcine liver microsomes and characterized (Lehner and Verger, 1997). TGH accounts for approximately 70% of hepatic microsomal lipolytic activity. The purified protein was isolated by solubilization by the zwitterionic detergent, CHAPS and subsequent column chromatography to yield a single protein band with an apparent molecular mass of ~60 kDa in SDS-PAGE. TGH hydrolyzes long-, medium- and short-chain TGs at the sn-1 and sn-3 positions (see Figure 1-1). It does not hydrolyze phospholipids or acyl-CoA thioesters. Divalent cations are not required for optimal lipolytic activity. The enzyme has a neutral pH optimum and is inactivated by the lipase inhibitors tetrahydrolipstatin, diethyl-p-nitrophenyl phosphate (E600) and diisopropyl fluorophosphates, indicating that it is a serine esterase (Lehner et al., 1999; Lehner and Verger, 1997). Amino-terminal

sequences of purified porcine TGH were found to be identical to that of porcine proline-β-naphthylamidase (Matsushima et al., 1991). TGH expression was immunodetected in livers from humans, rats, mice, hamsters and cows (Lehner et al., 1999). TGH was subsequently purified from human and mouse liver microsomes, and our laboratory has isolated cDNAs for rat, mouse and human TGH (Alam et al., 2002a; Dolinsky et al., 2001; Lehner and Vance, 1999). These cDNAs are predicted to encode proteins of 565 amino acids for rats and mice and 568 amino acids for humans (Figure 1-8). In addition to mobility on SDS-PAGE, the calculated molecular masses of these proteins are also ~60 kDa from the amino acid sequences derived from the cDNAs. The mouse and human TGH proteins share 92% identity and rat and human proteins share 93% identity at the amino acid level (Dolinsky et al., 2004b).

In rodents, TGH is highly expressed in liver and adipose tissue with lesser levels found in heart, kidney and small intestine, while in humans TGH is mainly expressed in the liver, adipose and small intestine (Dolinsky *et al.*, 2004b). TGH hydrolyzes stored TG and, in the liver, the lipolytic products are made available for VLDL synthesis. A similar function has been hypothesized for TGH in the small intestine regarding chylomicron assembly, although this remains to be demonstrated. A role for TGH in basal TG lipolysis in adipocytes has been described (Soni *et al.*, 2004). Background information regarding TGH is presented below, while data surrounding TGH that was generated for this Thesis are given in subsequent Chapters.

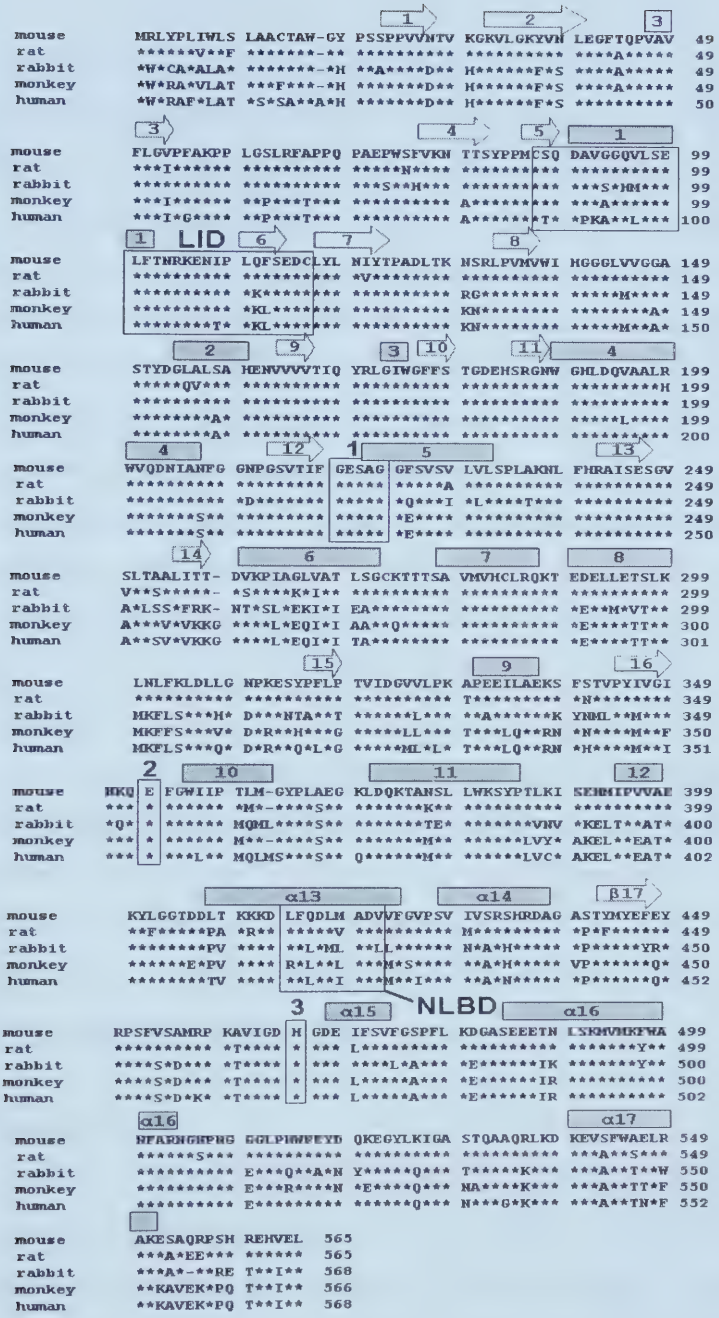
mouse MRLYPLIWLS LAACTAW-GY PSSPPVVNTV KGKVLGKYVN LEGFTQPVAV 49
rat *****V***F ********-** ********** ********** ****A***** 49
rabbit *W*CA*ALA* ********-*H **A****D** H*******F*S ****A***** 49
monkey *W*RA*VLAT ***F***-*H ********D** H*******F*S ****A***** 49
human *W*RAF*LAT *S*SA**A*H *******D** H*******F*S ********** 50
mouse FLGVPFAKPP LGSLRFAPPQ PAEPWSFVKN TTSYPPMCSQ DAVGGQVLSE 99
rat ***I****** ********** *****N**** ********** ********** 99
rabbit ********** ********** ***S**H*** ********** ***S*HM*** 99
monkey ***I****** **P***T*** ********** A********** ***A****** 99
human ***I*G**** **P***T*** ********** A********T* *PKA**L*** 100
LID
mouse LFTNRKENIP LQFSEDCLYL NIYTPADLTK NSRLPVMVWI HGGGLVVGGA 149
rat ********** ********** *V********* ********** ********** 149
rabbit ********** *K********* ********** RG******** *****M**** 149
monkey ********** *KL******** ********** KN******** ********A* 149
human ********T* *KL******** ********** KN******** *****M**A* 150
mouse STYDGLALSA HENVVVVTIQ YRLGIWGFFS TGDEHSRGNW GHLDQVAALR 199
rat *****QV**** ********** ********** ********** *********H 199
rabbit ********** ********** ********** ********** ********** 199
monkey ********A* ********** ********** ********** *****L**** 199
human ********A* ********** ********** ********** **********A* 200
mouse WVQDNIANFG GNPGSVTIF GESAG GFSVSV LVLSPLAKNL FHRAISESGV 249
rat ********** ********** ***** *****A ********** ********** 249
rabbit ********** *D******** ***** *Q***I *L****T*** ********** 249
monkey *******S** ********** ***** *E***** ********** ********** 249
human *******S** ********** ***** *E***** ********** ********** 250
mouse SLTAALITT- DVKPIAGLVAT LSGCKTTTSA VMVHCLRQKT EDELLETSLK 299
rat V**S*****- *S****K*I** ********** ********** ********** 299
rabbit A*LSS*FRK- NT*SL*EKI*I EA******** ********** *E**M*VT** 299
monkey A***V*VKKG ****L*EQI*I AA**Q***** ********** *E****TT** 300
human A**SV*VKKG ****L*EQI*I TA******** ********** *E****TT** 301
mouse LNLFKLDLLG NPKESYPFLP TVIDGVVLPK APEEILAEKS FSTVPYIVGI 349
rat ********** ********** ********** T********** *N********* 349
rabbit MKFLS***M* D***NTA**T ******L*** **A******K YNML**M*** 349
monkey MKFFS***V* D*R**H***G *****LL*** T***LQ**RN *N****M**F 350
human MKFLS***Q* D*R**Q*L*G *****ML*L* T***LQ**RN *H****M**I 351
mouse HKQ E FGWIIP TLM-GYPLAEG KLDQKTANSL LWKSYPTLKI SENMIPVVAE 399
rat *** * ****** *M*-****S** *******K** ********** ********** 399
rabbit *Q* * ****** MQML****S** *******TE* *******VNV *KELT**AT* 400
monkey *** * ****** M**-****S** *******M** ******LVY* AKEL**EAT* 400
human *** * ***L** MQLMS***S** Q******M** ******LVC* AKEL**EAT* 402
α13 α14 β17
mouse KYLGGTDDLT KKKD LFQDLM ADVVFGVPSV IVSRSHRDAG ASTYMYEFEY 449
rat **F*****PA *R** *****V ********** M********** *P*F******* 449
rabbit ********PV **** **L*ML **LL****** N*A*H***** *P******YR* 450
monkey ******E*PV **** R*L**L ***M*S**** **A*H***** VP*******Q* 450
human *********TV **** **L**I ***M**I*** **A*N***** *P*******Q* 452
NLBD
α15 α16
mouse RPSFVSAMRP KAVIGD H GDE IFSVFGSPFL KDGASEEETN LSKMVMKFWA 499
rat ********** *T**** * *** L********* ********** ********Y** 499
rabbit ****S*D*** *T**** * *** ****L*A*** *E******IK ********Y** 500
monkey ****S*D*** *T**** * *** L*****A*** *E*******IR ********** 500
human ****S*D*K* *T**** * *** L*****A*** *E*******IR ********** 502
α16 α17
mouse NFARNGHPNG GGLPIWFEYD QKEGYLKIGA STQAAQRLKD KEVSFWAELR 549
rat ******S*** ********** ********** ********** ***A**S*** 549
rabbit ********** E***Q**A*N Y*****Q*** T*****K*** ***A**T**W 550
monkey ********** E***R****N *E****Q*** NA****K*** ***A**TT*F 550
human ********** E********* ******Q*** N***G*K*** ***A**TN*F 552
mouse AKESAQRPSH REHVEL 565
rat ***A*EE**** ****** 565
rabbit ***A*-**RE T**I** 568
monkey **KAVEK*PQ T**I** 566
human **KAVEK*PQ T**I** 568

Figure 1-8 Amino acid sequences of TGH from various species. Residues identical to mouse TGH are represented by an asterisk. Arrows indicate regions predicted to form β-sheets in the mouse protein; rectangles indicate predicted α-helices. Boxed residues indicate a lid domain (that may protect the active site and regulate substrate specificity and entry to the active site) and a putative neutral lipid binding domain (NLBD), **1**) GXSXG catalytic serine motif **2**) catalytic glutamic acid **3**) catalytic histidine

1.4.2 TGH belongs to the carboxylesterase family of enzymes

Sequence analysis showed that TGH has minimal amino acid identity to previously identified lipases, and is more closely related to the family of mammalian carboxylesterases (E.C. 3.1.1.1) (Dolinsky *et al.*, 2004b). These enzymes are characterized by their ability to hydrolyze ester (including lipid and retinyl ester), thioester, or amide bonds (Harrison, 2000; Satoh and Hosokawa, 1998). Because of their ability to address these various types of chemical bonds, roles in drug and xenobiotic metabolism and detoxification have been illuminated (Satoh and Hosokawa, 1998). Several isoforms of carboxylesterases have been identified in mammalian tissues. These enzymes have been shown to have distinct biochemical, immunological and genetic properties (Satoh and Hosokawa, 1998). Some carboxylesterases were also shown to bind and retain proteins within a specific subcellular compartment (Lee *et al.*, 2000; Pfister *et al.*, 1988; Yue *et al.*, 1996).

Given the number of different carboxylesterase proteins that have been described by different groups, a confusing number of names have been given to the TGH protein. For example, the carboxylesterase protein that is identical to rat TGH has been referred to as RL1 (Hosokawa *et al.*, 1987), ES10/pI 6.1 esterase (Robbi *et al.*, 1990), hydrolase A (Morgan *et al.*, 1994) and neutral cholesterol esterase (Ghosh *et al.*, 1995).

1.4.3 Localization of TGH

If TGH activity were a component of the pathway for assembly and secretion of apoB containing lipoprotein particles, then it would be anticipated that TGH would be expressed in tissues that have the ability to synthesize and secrete apoB containing lipoproteins. The high level of TGH expression in the liver is consistent with its involvement in the mobilization of intracellular TG stores for VLDL secretion (Dolinsky *et al.*, 2004b; Gilham and Lehner, 2004). Immunocytochemical studies localize TGH expression exclusively to adult hepatocytes surrounding the capillary vessels leading to the central vein (Lehner

et al., 1999). This region of the liver is most likely to be active in lipoprotein production and secretion. TGH co-sediments with ER and mitochondria-associated membranes (Lehner *et al.*, 1999), which have been demonstrated to contain enzymatic activities required for synthesis and assembly of lipoproteins (Rusinol *et al.*, 1994). Rat TGH could only be detected in liver parenchymal cells, but not Kupffer or endothelial cells (Gaustad *et al.*, 1992).

1.4.4 Structural motifs and consensus sequences in TGH

The porcine TGH protein has an N-terminal 18 amino acid signal peptide that directs protein synthesis into the lumen of the ER where TGH has been immunodetected (Lehner *et al.*, 1999). The signal peptide is removed once inside the lumen by an ER resident signal peptidase, as indicated by N-terminal sequencing of the purified protein (Lehner and Verger, 1997). These results were confirmed by collaborators at GlaxoSmithKine in completely independent experiments.

The primary sequence of human TGH contains a putative tyrosine phosphorylation site at Tyr118, as well as seven potential serine/threonine consensus sequences for phosphorylation by casein kinase II. The Tyr118 site is adjacent to the lid domain formed by the loop created by a disulfide bridge between Cys87 and Cys116, potentially regulating the opening of the lid and entry of substrate into the catalytic cleft and the active site. Phosphorylation of TGH is the focus of a collection of post-translational modification studies described in Chapter 6.

Two consensus sequences for N-linked glycosylation have been identified at Asn79 and Asn489 in the mouse and rat TGH while the human protein contains a single potential glycosylation site at Asn79 (Dolinsky *et al.*, 2004b). A requirement for glycosylation of human TGH in protein folding and catalysis is investigated in Chapter 6. The mature sequence of mouse TGH contains four Cys residues while the human TGH encodes an additional Cys at residue 390. A further post-translational modification that has been observed on carboxylesterases is acylation. Fatty acylation of a porcine liver

carboxylesterase has been recently suggested (Smialowski-Fleter *et al.*, 2002). Palmitoylation occurs post-translationally on free cysteine residues via a labile thioester bond. Unlike myristoylation, palmitoylation is readily reversible. There is a possibility that the free Cys390 residue of human TGH undergoes reversible palmitoylation, which could contribute to transiently increased affinity of TGH for hydrophobic surfaces like membranes or lipid storage droplets. The potential for this postraslational modification to TGH is investigated in Chapter 6.

1.4.5 Proposed structure of the TGH protein

Analysis of the mouse and human TGH protein sequences with PSI-Pred and Predict Protein secondary structure prediction programs rendered a succession of strong and well defined predictions for 17 α-helices and 17 β-strands throughout the amino acid sequence (Figure 1-8). In addition, these elements are strongly conserved among orthologous carboxylesterases from mouse, rat, rabbit, monkey and human (Figure 1-8) (Dolinsky *et al.*, 2004b). TGH contains a GXSXG motif, which is found in all lipases and houses the catalytic serine residue (Lehner and Verger, 1997). The remaining amino acid residues in the human protein involved in a catalytic triad have been subsequently determined by site-directed mutagenesis and expression of the mutants using a baculovirus expression system (Alam *et al.*, 2002b). Alignment of amino acid sequences identify these critical residues in TGH from the other species (Figure 1-8). The Ser221 (221), Glu353 (354) and His466 (468) mouse (human) residues form the TGH catalytic triad and are highly conserved between species.

A three-dimensional model for human TGH has been proposed based on the crystal structure co-ordinates of acetylcholine esterase and overlapping active sites with pancreatic lipase (Alam *et al.*, 2002b) (Figure 1-9). The modeled structure shares the overall folding and topology of the proteins identified in the recently published crystal structures of the rabbit (Bencharit *et al.*, 2002) and human (Bencharit *et al.*, 2003a; Bencharit *et al.*, 2003b) esterase (Es) 22 (also known as egasyn) proteins. Several distinct structural motifs are apparent. In

general, the structure of TGH may be viewed as being comprised of a central catalytic domain surrounded by regulatory domains. TGH has a three-dimensional α/β hydrolase fold that is a structural feature of all lipases (Wong and Schotz, 2002). In essence, the α/β hydrolase fold consists of a central β-sheet surrounded by a variable number of α-helices. This structural element accommodates the catalytic triad composed of Ser, His and a carboxylic acid. The catalytic triad is located at the bottom of a deep active site cleft approximately in the center of the molecule, and is comprised of a large flexible pocket on one side of the catalytic serine and a small rigid pocket on the opposite side. The residues that compose the catalytic domain of human TGH are very highly conserved among TGH proteins from different species (Figure 1-8). The orientation and location of the active site provides an ideal hydrophobic environment for the hydrolysis of a wide variety of hydrophobic substrates.

The small rigid active site pocket is adjacent to the oxyanion hole formed by Gly142/143 and is lined by several hydrophobic residues. Short acyl-chains would be easily accommodated within tne small rigid pocket. The larger flexible active site pocket is lined by several non-polar residues and could accommodate larger or polycyclic molecules such as cholesterol. The large pocket is adjacent to a side-door secondary pore that would permit small molecules (substrates and reaction products) to enter and exit the active site (Bencharit *et al.*, 2003b). Longer acyl-chains may be oriented for catalysis in such a way that they extend through the side door. Indeed the presence of a hydrophobic residue at position 423 in mice and 425 in humans is necessary for efficient hydrolysis of hydrophobic substrates as mutation of Met present in position 423 of the related rat lung carboxylesterase to Ile increased the carboxylesterase activity towards a more hydrophobic substrate without affecting activity towards short-chain esters (Wallace *et al.*, 2001). According to the X-ray crystal structure of human Es 22, this residue lines the flexible pocket adjacent to the side door. Given the wide range of substrates that carboxylesterases are known to hydrolyze, the large flexible pocket confers the ability to hydrolyze many structurally distinct

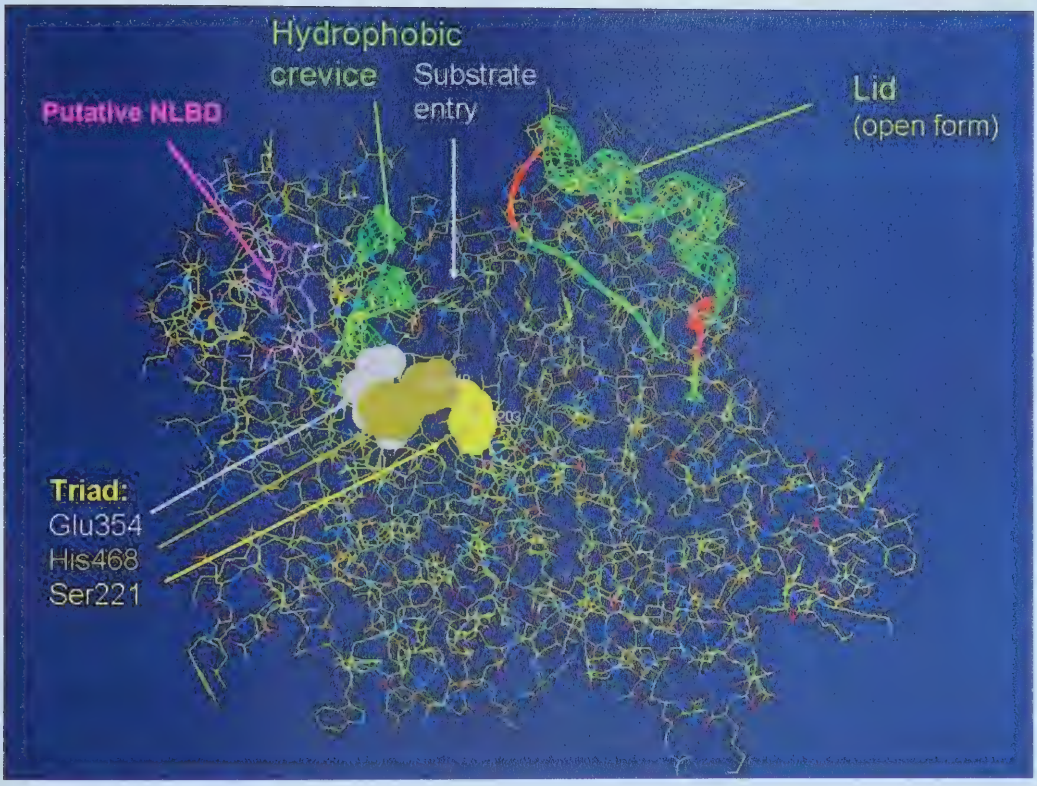

Figure 1-9 Three dimensional model of human TGH. The model was developed based on the coordinates obtained from crystal structures of acetylcholine esterase and pancreatic lipase. TGH is essentially clam shaped, with a hydrophobic crevice allowing access to the active site and the catalytic triad. NLBD, putative neutral lipid binding domain.

compounds, whereas the rigid pocket is much more selective with regard to the substrates that may be accommodated.

The entrance to the active site cleft of human TGH is surrounded by several α-helices that define the regulatory domain that controls access of substrates to the TGH active site. Alpha helix 1 closes over the active site cleft and acts as a lid (this is indicated in the three dimensional model in Figure 1-9, and the primary amino acid sequence of the lid is indicated in Figure 1-8 and 1-10). The amino acid sequence of α-helix 1 is highly conserved among orthologous TGH proteins, suggesting that it is necessary for TGH activity (Figure 1-8). The lid domain plays a vital role in lipolysis at the lipid-water interface by interfacial activation. In the absence of lipid-water interface the active site is covered by the "lid". In the presence of hydrophobic substances the "lid" is opened and the catalytic site becomes accessible to the substrate. Interfacial activation kinetics were probed for both porcine and human TGH (Alam *et al.*, 2002a; Lehner and Verger, 1997). The adjacent α-helix 13 contains a putative neutral lipid binding domain (NLBD) that could potentially mediate the affinity TGH has for lipid surfaces or substrate molecules (Figures 1-8, 1-9 and 1-10). The FLXLXXXn (n=nonpolar amino acid residue) motif is present in several proteins that bind lipids, and is conserved among the orthologous TGH proteins currently identified (Figure 1-8). Studies that dissect the NLBD in TGH and other proteins that carry this domain remain undescribed.

In addition to its ability to hydrolyze lipids, TGH has been implicated in xenobiotic metabolism. TGH is one of a number of hepatic enzymes that can synthesize fatty acid ethyl esters *in vitro*, which are toxic metabolites of chronic alcohol consumption (Diczfalusy *et al.*, 2001; Pfutzer *et al.*, 2002; Tsujita and Okuda, 1992), as well as generate the toxic cocaine metabolite, cocaethylene that is formed when cocaine and alcohol are taken together (Brzezinski *et al.*, 1994). A great deal of effort has been placed on enhancing the cytotoxicity of the anti-tumor pro-drug, irinotecan. The *in vivo* activation of irinotecan to the topoisomerase I inhibitor, SN-38 (ethyl-10-hydroxycamptothecin) is catalyzed by carboxylesterases. Overexpression of human and mouse TGH resulted in

increased activation of irinotecan to SN-38 and enhanced cytotoxicity in a variety of tumor cell lines (Humerickhouse *et al.*, 2000; Wu *et al.*, 2002; Xie *et al.*, 2003). However, these studies also showed that there are hepatic carboxylesterases with higher irinotecan hydrolytic activity than TGH.

1.4.6 Location and structure of the TGH gene

The TGH genes are located within a cluster of carboxylesterase genes on mouse and human chromosomes 8 and 16, respectively. Both the mouse and human TGH genes span approximately 30 kb and contain 14 exons (Dolinsky *et al.*, 2004b). Recently, the sequencing of the mouse and human genomes were completed enabling detailed sequence comparisons. The previously published sequences of the individual exons, splice junctions, size of the introns and restriction sites within the mouse and human TGH genes are consistent with their respective genes sequenced by the mouse and human genome projects. Thus the organization of the TGH gene is evolutionarily conserved between mice and humans. Previous studies have mapped the human carboxylesterase gene to chromosome 16 at 16q13-q22.1 (Kroetz *et al.*, 1993; Zschunke *et al.*, 1991). This region is syntenic to a region of mouse chromosome 8 at 8C5 (Zschunke *et al.*, 1991). The mouse carboxylesterases Es 22 (Eisenhardt and von Deimling, 1982; Ovnic *et al.*, 1991a) and Es1 (Ovnic *et al.*, 1991b; Womack, 1975) have been previously mapped to chromosome 8. The completion of the mouse genome sequencing project unambiguously demonstrated that mouse TGH gene was located on the minus strand of chromosome 8 at 8C5 in a cluster of six carboxylesterase genes that spans 260.6 kb in total (Dolinsky *et al.*, 2004b). These six carboxylesterase genes are presumed to have originated from repeated gene duplications of a common ancestral gene that encoded a carboxylesterase and subsequent evolutionary divergence occurred. Evidence for their common ancestral origin comes from the shared structure and size of these genes. The proteins encoded by Es 22 (Ovnic *et al.*, 1991a) and CES1 (Ellinghaus *et al.*, 1998) are most closely related, having 76% identity to mouse TGH, while M-LK has 70% (Xie *et al.*, 2003) and Es1 (Ovnic *et al.*, 1991b) has

69% identity to TGH (Figure 1-10). A sixth gene, 2310039D24 Rik does not code for a complete carboxylesterase protein. 2310039D24 Rik has identity to the 305 C-terminal residues of mouse TGH, but does not contain the GXSXG catalytic serine motif. If this protein is expressed in a cell, it would not be expected to have esterase activity, and its putative function is obscure. The Ser, Glu and His catalytic residues were conserved in the remaining five carboxylesterase genes in the cluster, suggesting that they all have esterase activity (Satoh and Hosokawa, 1998). Furthermore, it appeared that most of the secondary structural features were also conserved. This indicates that the tertiary structure of the proteins encoded by genes within the carboxylesterase cluster are likely very similar, suggesting they could all hydrolyze similar substrates.

The ES1 gene is composed of 13 exons and 12 introns. It lacks an exon that encodes a loop and β-sheet that form a portion of the active site cleft in the structure of TGH (Bencharit *et al.*, 2003b). It is predicted that ES1 has a smaller active site cleft, making it much more selective towards substrates that may be accommodated within this altered active site compared to the other carboxylesterases encoded by the gene cluster. In addition, the C-terminus of ES1 lacks an ER-retrieval sequence, resulting in secretion of the ES1 protein (Murakami *et al.*, 1993). The most diverged region among the proteins encoded by the genes in the carboxylesterase cluster is located in the regulatory domain α-helix 13. As indicated above, TGH contains a putative NLBD in α-helix 13 (Section 1.4.5). The variability of amino acids in this regulatory domain may be responsible for the differences in substrate selectivity and inhibition profiles of carboxylesterases encoded by genes in this cluster. At the moment, there is no experimental evidence that any of these carboxylesterases, besides TGH, hydrolyze lipid substrates or mobilize TG stored within the liver for secretion.

Figure 1-10 Amino acid sequences of proteins encoded in a mouse carboxylesterase gene cluster. The mouse TGH amino acid sequence is shown at the top. Identical residues to TGH are represented with an asterisk. Boxes highlight noteworthy domains or residues including a lid region that may regulate substrate specificity and entry to the active site. **1**) GXSXG catalytic serine motif **2**) catalytic glutamic acid **3**) catalytic histidine and a putative neutral lipid binding domain (NLBD).

1.4.7 The TGH promoter

TGH expression and activity is both developmentally and hormonally regulated. To investigate the transcriptional regulation of the TGH gene, human and mouse TGH proximal promoters were isolated. The mouse TGH promoter sequence (Douglas *et al.*, 2001) shares 46% identity to the human (Langmann *et al.*, 1997). The promoters share several common binding sites for transcription factors (Figure 1-11), suggesting that the orthologous TGH genes have evolutionarily conserved transcriptional regulatory patterns. A TATA box does not precede the transcription start site. Potential binding sites for transcription factors found in both promoters include Sp1 binding sites, glucocorticoid response elements half-sites and binding sites for CCAAT enhancer binding protein (C/EBP-β). Despite these similarities, differences for potential binding sites (and the order in which that appear) allow the speculation that functional differences in the expression of mouse versus human TGH occur.

Both the TGH mRNA and protein are expressed in mouse and rat liver toward the end of the suckling period (Douglas *et al.*, 2001; Lehner *et al.*, 1999), which coincides with ontogeny of VLDL secretion. This altered TGH expression appears to be related to dietary changes at the time of weaning and independent of hepatic differentiation because TGH expression was unchanged in regenerating livers that undergo de-differentiation and acquire fetal and neonatal features following partial hepatectomy (Lehner *et al.*, 1999). The transcription factor Sp1 has been implicated in the dramatic increase in hepatic TGH mRNA and protein observed during the suckling / weaning transition in mice (Douglas *et al.*, 2001).

TGH expression is induced in 3T3-L1 cells during differentiation (Dolinsky *et al.*, 2003). It has been shown that TGH mRNA expression is upregulated by CAAT enhancer-binding protein-α (C/EBPα) in 3T3-L1 cells during and after the differentiation process (Wei *et al.*, 2005).

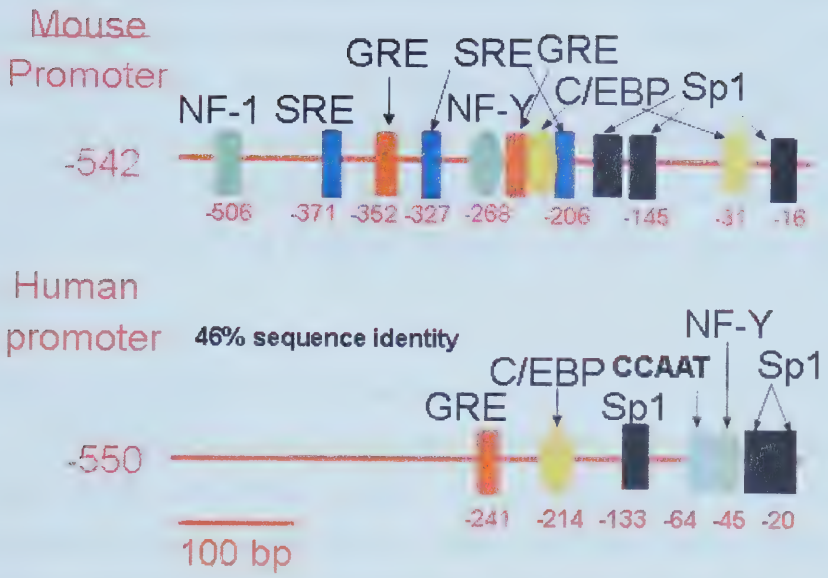

Figure 1-11 Putative transcription factor binding sites in the 5' flanking region of the triacylglycerol hydrolase gene. Sequences for the mouse and human TGH promoters were obtained from the respective genome sequencing projects. The promoters were analyzed for potential binding sites for known transcription factors using the TRANSFAC database. Blue boxes= sterol response elements (SRE), blue circles= CCAAT box, red boxes= glucocorticoid response elements half-sites (GRE), green boxes= NF-1, green circles= NF-Y, black boxes= Sp1, and yellow circles= CCAAT enhancer binding protein (C/EBP-β)

1.4.8 Mechanism for TGH participation in VLDL assembly

In our model of TG mobilization for VLDL assembly, TGH acts to hydrolyze TG in lipid storage droplets that are associated with the ER (Figure 1-12). The lumenal lipid droplet is derived from cytoplasmic stores in a process that involves MTP. Lipolysis of TG to DG/MG would make the lipolytic products more soluble in the membrane, thereby facilitating the efficient transfer of the acylglycerols for resynthesis to TG by lumenally oriented acyltransferases and availability to a developing apoB containing entity. TG that is not assembled onto apoB may be returned to either cytosolic or lumenal storage pools in a futile cycle.

1.4.9 Lipid droplets: Endogenous substrate for TGH

Most cells in the body can synthesize and store neutral lipids as intracellular droplets (Murphy, 2001; Murphy and Vance, 1999), however, only a few tissues have the ability to store large quantities of neutral lipids. Intracellular lipid droplets, much like lipoprotein particles, are spherical and are composed of a hydrophobic core of TG and CE surrounded by a monolayer of phospholipids and sterols. In addition, lipid droplets are surrounded by a specific population of proteins that may be involved in lipid droplet synthesis, trafficking, stabilization and lipolysis, such as perilipin, adipophilin (the mouse ortholog is termed adipose differentiation-related protein (ADRP)) and tail interacting protein of 47 kDa (TIP47) (Londos *et al.*, 1999b; Miura *et al.*, 2002; Wolins *et al.*, 2005; Wolins *et al.*, 2001).

1.4.10 Use of hepatoma cells to investigate VLDL assembly

Much of our understanding of the mechanism of VLDL assembly and secretion is derived from cultured cells. HepG2 cells are derived from a human hepatoma. They are unusual in that they secrete few VLDL-sized particles, but rather secrete apoB in the LDL-HDL density range (Boren *et al.*, 1993; Gibbons *et al.*, 1994; Wu *et al.*, 1996). Gibbons *et al.* (Gibbons *et al.*, 1994) suggested

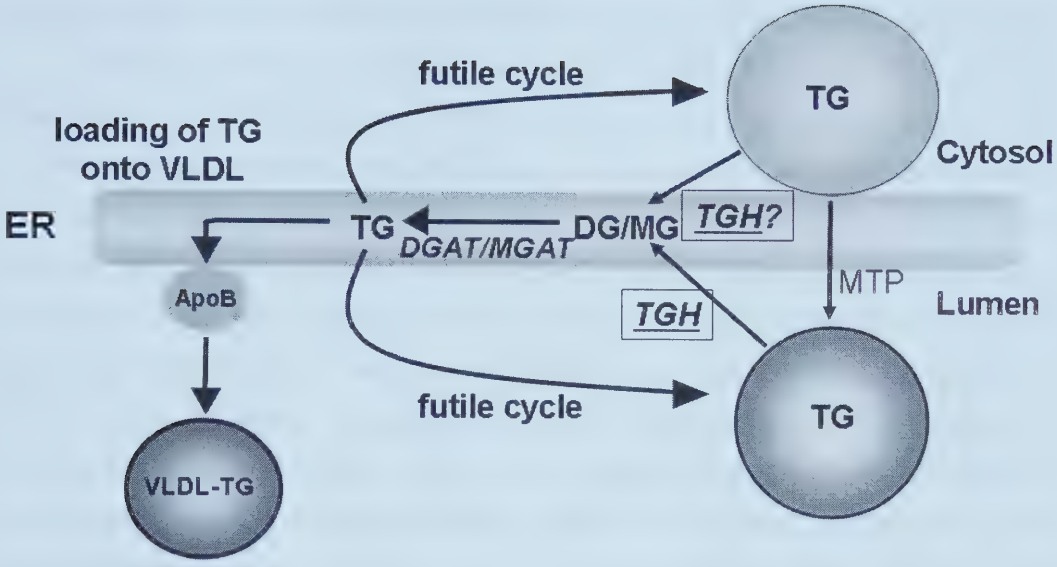

Figure 1-12 Model of triacylglycerol (TG) mobilization for VLDL assembly. TG from cytosolic storage pools is transferred to a droplet within the lumen of the endoplasmic reticulum (ER) via the action of microsomal triglyceride transfer protein (MTP). The lumenal droplet may be the primary source of TG for very low density lipoproteins (VLDL). The lipid is mobilized from this droplet by TG hydrolase (TGH) catalyzed lipolysis to partial acylglycerols like diacylglycerol (DG) and fatty acid. Reacylation of these products back to TG would occur via lumenally oriented acyltransferases such as di- or monoacylglycerol acyltransferase (DGAT/MGAT). The TG would then be available for loading onto a nascent apoB containing particle. TG not utilized for VLDL assembly is returned to the storage pool (futile cycle). Alternately, TGH may access TG at the inner ER membrane. Upon contact with the ER, TGs could egress from the cytosolic lipid droplet into the ER bilayer where they could encounter TGH.

that the impaired secretion of VLDL from HepG2 cells is due to a defect in the mobilization of TG from storage pools. This conclusion was supported by studies by Wu *et al.* (Wu *et al.*, 1996) who demonstrated a similar inability of stored TG to be mobilized from HepG2 cells for assembly into apoB containing lipoproteins.

McArdle RH7777 cells are a rat hepatoma cell line. They require incubation with fatty acids in order to secrete lipids, unlike hepatocytes that continue to secrete lipids after fatty acids are withdrawn from the media (Lehner and Vance, 1999). Similar to HepG2 cells, studies have shown that compared to primary hepatocytes, McArdle RH7777 cells are also deficient in the mobilization of stored TG for VLDL assembly and secretion (Lehner and Vance, 1999). It is likely that fatty acids are trapped in intracellular storage pools because both of these cell lines (HepG2 and McArdle RH7777) do not express TGH (Lehner *et al.*, 1999; Lehner and Vance, 1999). The hepatoma cell lines McArdle RH7777 and HepG2 primarily secrete TG that is derived from newly synthesized TG and are deficient in the mobilization of TG from the intracellular storage pools (Gibbons *et al.*, 1994; Lehner and Vance, 1999). This may account for the observed differences between the mechanism and capacity of hepatoma cells and primary hepatocytes to secrete VLDL.

1.4.11 Transfection of McArdle RH7777 cells with TGH

To gain further insight into the function of TGH, our laboratory generated McArdle RH7777 cell lines that stably express rat TGH (Lehner and Vance, 1999). Stable transfection resulted in depletion of the intracellular TG stores at a level 2-fold higher than empty-vector transfected cells (Lehner and Vance, 1999). TGH-transfected cells also secreted an approximately 25% greater mass of TG into the medium and had increased levels of apoB in the VLDL density range. Phosphatidylcholine secretion was essentially the same in control and TGH-transfected cells. Studies in TGH-transfected cells demonstrated preference for directing the lipolytic products into the secretory pathway. The results suggest that microsomal TGH actively participates in the mobilization of TG from storage pools, some of which can be used for lipoprotein assembly.

1.4.12 Use of primary hepatocytes in neutral lipid research

The primary hepatocyte model is a well-characterized model for measuring the contribution of the lipolysis and re-esterification pathway to TG secretion (Gibbons *et al.*, 2000; Wiggins and Gibbons, 1992). Our laboratory determined by RT-PCR whether or not cultured primary mouse hepatocytes express mRNAs for DGAT, TGH and other carboxylesterase enzymes that may be involved in TG lipolysis and re-esterification, and followed the length of time in culture that hepatocytes maintained the expression of these mRNAs (Dolinsky *et al.*, 2004b). DGAT-1 mRNA expression could be detected up to 72 h in cell culture, but declined after 36 h. DGAT-2 mRNA expression could be detected in hepatocytes up to 42 h in culture, but markedly declined after 30 h in culture. TGH mRNA is expressed for 72 h, but declines after 48 h. The other closely related carboxylesterases, Es 22 and CES1 were highly expressed in hepatocyte culture for 24 h, after which their expression declined until it was no longer detectable beyond 48 h. Therefore it has been established that primary hepatocytes are an appropriate model for measuring intracellular TG lipolysis/re-esterification up to 30 h in culture. This work is consistent with experiments of Bartlett and Gibbons that demonstrated that the rate of TG secretion by primary rat hepatocytes is linear for the first 24 h of culture and then declines markedly up to 72 h (Bartlett and Gibbons, 1988). This model was therefore employed in a number of experiments described in this Thesis.

1.4.13 Regulation of TGH expression by dietary fatty acids and sterols

Both the degree of fatty acid saturation and the addition of cholesterol to a high fat diet modify the impact of the diet on lipid homeostasis. Our laboratory examined whether a diet enriched with fat altered hepatic TGH expression. TGH expression was not affected by supplementation of the diet with 20% fatty acid, regardless of the degree of saturation (Dolinsky *et al.*, 2003). Therefore, it appears that a high fat diet does not alter the level of hepatic TGH-mediated lipolysis. Including cholesterol with the high fat diet, however, increased hepatic

TGH expression in mice approximately 2-fold. This induction of TGH expression is consistent with the observed induction of the orthologous human TGH expression by incubation of human macrophages with exogenous low density lipoproteins (Becker *et al.*, 1994; Langmann *et al.*, 1997). Although sequences that bear some similarity to sterol response elements exist in the TGH proximal promoter in mice, the functionality of these sequences has not been determined (Douglas *et al.*, 2001). Further, the enforced expression of a nuclear form of sterol regulatory element binding protein-1a in transgenic mice did not alter TGH mRNA expression in the liver (R. Lehner and D.E. Vance, unpublished observation). It is currently unclear whether the cholesterol mediated regulation of TGH expression is due to SREBPs, oxysterol nuclear receptors, or an indirect mechanism. It is important to note that hypercholesterolemic *ApoE*$^{-/-}$ mice also have a 3-fold higher level of hepatic TGH expression than *wild type* mice (Huang *et al.*, 2000), supporting a role for cholesterol in influencing TGH expression in liver.

1.5 Adipose TG storage and lipolysis

1.5.1 Adipose TG storage

White adipose tissue stores the largest quantities of TG, and forms intracellular neutral lipid droplets that may exceed 50 μm in diameter, though the size varies based on the nutritional status of the animal. Lipid storage within white adipose tissue serves as a reservoir for excess energy, as a heat insulator and is now recognized as an endocrine organ important to the regulation of energy intake through the secretion of hormones such as leptin (see Section 1.6.1) among others (Kim and Moustaid-Moussa, 2000). During metabolic excess, TG is stored in adipose; during metabolic requirement, free fatty acids are liberated and carried by serum albumin in the plasma for delivery to tissues for utilization (Frayn, 2002; Horowitz, 2003). It has been estimated that approximately 10% of stored TG within adipocytes undergoes turnover on a daily basis (Brooks *et al.*, 1982; Ookhtens *et al.*, 1987). Intracellular TG lipolysis may

be grouped into two classes: a well described hormonally regulated lipolysis (Section 1.5.2) and a relatively uncharacterized basal lipolysis (Section 1.5.3).

1.5.2 Hormone stimulated lipolysis in adipose

In the adipocyte, hormone stimulated lipolysis is catalyzed by HSL. HSL is recognized to catalyze both the hydrolysis of CE, as well as TG to DG and DG to MG (Fredrikson *et al.*, 1981; Lee *et al.*, 1988). A second enzyme, monoacylglycerol lipase catalyzes the final hydrolysis of MG to free glycerol and fatty acids (Karlsson *et al.*, 2001). Monoacylglycerol lipase has not been studied in detail, though it does not appear to be acutely regulated. On the other hand, HSL is rapidly activated by lipolytic hormones such as catecholamines and glucagon by a mechanism that involves a hormone/G-protein induced increase in cyclic adenosine monophosphate (cAMP) (Walsh *et al.*, 1968). The increased cAMP activates cAMP-dependent protein kinase (PKA) to phosphorylate HSL, resulting in increased lipolytic activity (Strålfors *et al.*, 1984). Phosphorylation of HSL not only increases the activity of the enzyme, but also causes HSL to translocate from the cytosol to the surface of lipid droplets (Clifford *et al.*, 2000). The mechanism of the lipolytic stimulation also involves the lipid droplet surface protein perilipin, which acts as a barrier in non-stimulated cells, preventing access of HSL to the lipid droplet (Londos *et al.*, 1995). Overexpression of perilipin in NIH-3T3 or 3T3-L1 fibroblasts promotes TG storage and decreases the rate of TG hydrolysis (Brasaemle *et al.*, 2000b; Souza *et al.*, 2002). PKA phosphorylates perilipin and stimulates lipolysis whereas mutation of the PKA phosphorylated sites on perilipin abolished the ability of lipolytic hormones to stimulate lipolysis. In addition, perilipin as well as HSL are dephosphorylated in response to insulin resulting in reduction of TG lipolysis. Targeted deletion of the mouse perilipin gene produced a lean animal with higher rates of basal lipolysis and blunted hormone stimulated lipolysis (Martinez-Botas *et al.*, 2000; Tansey *et al.*, 2001). Together these results demonstrated that the phosphorylation state of perilipin influences the ability of HSL to interact with the lipid droplet. Since over expression of perilipin caused HSL to be distributed throughout the cell, this

result was consistent with the hypothesis that perilipin regulates lipolytic activity by influencing the ability of HSL to access the lipid droplet.

1.5.3 Basal lipolysis in adipose

As indicated above, adipose tissue forms large intracellular TG droplets, and HSL is involved in mobilizing fatty acids from adipocyte TG stores. Targeted deletion of the *Hsl* gene in mice blunted the hormonal stimulation of TG lipolysis in adipose tissue both *in vitro* and *in vivo*. The body weight and cold tolerance of $Hsl^{-/-}$ mice were unchanged (Osuga *et al.*, 2000; Wang *et al.*, 2001). Surprisingly, disruption of the *Hsl* gene eliminated only 50% of the basal TG lipase activity of the adipose tissue, revealing that another TG lipase activity exists in adipocytes. Currently besides HSL, the only additional intracellular neutral TG lipases identified within the adipocyte are adipose triglyceride lipase (ATGL) (Zimmermann *et al.*, 2004) and TGH (Dolinsky *et al.*, 2001), suggesting involvement of TGH in the mobilization of fatty acids from adipocyte TG stores. Inhibition of TGH utilizing a specific enzyme inhibitor reduced basal free fatty acid release by 3T3-L1 adipocytes and circulating free fatty acid levels in hamsters by approximately 40% (W. Gao, and R. Lehner, unpublished results). Therefore, TGH may contribute a major portion of adipocyte TG basal lipolysis (Soni *et al.*, 2004).

1.6 Insights into hormonal influence on lipid metabolism

1.6.1 Leptin as a regulator of TG storage

The ability to communicate energy status to pathways regulating energy intake and expenditure is mediated by circulating hormones. Leptin is a peptide hormone of 16 kDa that is secreted by adipocytes and functions to regulate the size of lipid stores through multiple mechanisms acting on metabolic rate (Doring *et al.*, 1998; Geiser *et al.*, 1998; Halaas *et al.*, 1995; Mistry *et al.*, 1997; Stehling *et al.*, 1996), fuel selection (leptin reduces carbohydrate oxidation and increase fat utilization) (Hwa *et al.*, 1997) and food consumption (Campfield *et al.*, 1995; Halaas *et al.*, 1995; Pelleymounter *et al.*, 1995). Genetic disruption of the leptin

gene arose spontaneously in a mouse colony (Ingalls *et al.*, 1950). The animals are termed *ob/ob* mice, and they are strikingly obese. It is a very common model used in obesity research, particularly with respect to genes involved in achieving and obese state or strategies that reduce an obese condition. The leptin receptor is widely expressed, but is most highly expressed in the hypothalamus, which is a region of the brain central to influencing feeding behaviour (Lee *et al.*, 1996; Tartaglia *et al.*, 1995). In the absence of the leptin signal, the body does not sense that there are adequate fat stores, and in turn the organism becomes hyperphagic and obese. Plasma leptin levels correlate with adipose tissue mass and decrease after weight loss (Maffei *et al.*, 1995). Administration of leptin to *wild type* and *ob/ob* mice resulted in dose dependent decreases in body weight (Halaas *et al.*, 1997). Based on these observations, the potential of leptin as a pharmacological anti-obesity agent was intensely pursued. Unfortunately, resistance to this hormone is common amongst obese individuals, and further, administration of therapeutic leptin promoted resistance (Van Heek *et al.*, 1997). Leptin appears to decrease lipogenesis (Bai *et al.*, 1996; Wang *et al.*, 1999a) and stimulate lipolysis (Fruhbeck *et al.*, 1998; Fruhbeck *et al.*, 1997; Fruhbeck *et al.*, 2001; Wang *et al.*, 1999a).

1.6.2 Peroxisome proliferators activated receptors influence lipid metabolism

Regulating the mRNA expression of genes involved in lipid metabolism provides a mechanism to regulate levels of TG storage. Peroxisome proliferator-activated receptors (PPARs) are nuclear hormone receptors that utilize poly-unsaturated fatty acids as natural ligands and regulate the expression of a range of genes involved in lipid metabolism (Desvergne and Wahli, 1999; Ferre, 2004; Lee *et al.*, 2003). Three related PPAR isotypes have been identified in vertebrates and were named PPARα, PPARδ and PPARγ. Several synthetic ligands for the PPARs have been developed as potent activators. Fibrates are commonly used clinically as activators of PPARα whereas thiazolinediones (TZDs) are used as activators of PPARγ (Staels *et al.*, 1998). PPARα agonists, such as the fibrates, have beneficial effects on the plasma lipid profile through

the increased oxidation of fatty acids, decreased circulating TG and reduced hepatic and adipose tissue TG storage (Staels *et al.*, 1998).

In adult rodents, PPARα is expressed in all tissues that were tested with highest levels in brown adipose, liver, kidney, heart and duodenum (Braissant *et al.*, 1996; Lemberger *et al.*, 1996). Therefore, PPARα expression correlates with tissues that have a high capacity for mitochondrial and peroxisomal β-oxidation. Administration of fibrates to rodents resulted in numerous hepatic alterations. These included increased expression of mitochondrial and peroxisomal fatty acid metabolizing genes, as well as increased oxidation of fatty acids, decreased circulating TG and reduced adipose tissue TG stores (Staels *et al.*, 1998). The decreased circulating TG was a consequence of increased VLDL clearance from the circulation by increased lipoprotein lipase (LpL) activity and reduced apoCIII expression (Kesaniemi and Grundy, 1984; Schoonjans *et al.*, 1996). LpL is a TG lipase found on the endothelium of capillaries. ApoCIII inhibits binding of lipoproteins to the endothelial surface and lipolysis by LpL. PPARα null mice have lower levels of hepatic expression of mitochondria genes including, very long chain and long chain acyl-CoA dehydrogenase, long chain acyl-CoA synthetase and 3-ketoacyl-CoA thiolase, compared to *wild type* mice (Akiyama *et al.*, 2001; Aoyama *et al.*, 1998). These changes are accompanied by marked hepatic lipid accumulation and inability to increase ketone body production in response to fasting, suggesting that fatty acid oxidation is impaired. Collectively it is clear that PPARα is a critical mediator of lipid homeostasis.

PPARγ appears to be a key regulator of adipogenesis. PPARγ has a restricted pattern of expression, with highest levels in white and brown adipose tissues. Lower levels are detected in the small intestine, skeletal muscle, liver and heart. The development of the insulin-sensitizing drugs TZDs represents a significant advance in antidiabetic therapy. Type II diabetes is a chronic disease that affects 5–10% of adults over the age of 30 in most populations (Thiemermann, 2004). Several TZD compounds are now available for clinical use for reducing peripheral insulin resistance. Rosiglitazone is the most potent and selective PPAR-γ agonist to date, and there is a good correlation between

the potency of the TZDs as PPAR-γ agonist *in vitro* and their efficacy at lowering glucose levels *in vivo*. The beneficial effects of TZDs are not limited to insulin-sensitizing in type II diabetes, but can be observed in a multitude of diseases associated with ischemia–reperfusion and inflammation (Khandoudi *et al.*, 2002). With the involvement of PPARs in diverse metabolic pathways, there is intense clinical interest in the utility of PPAR ligands for the treatment of cancer, inflammation, psoriasis, atherosclerosis, dyslipidaemia, neurological disorders, obesity and diabetes.

The targeted deletion of the PPARγ gene is embryonic lethal as placental development is defective (Chawla *et al.*, 1994; Kletzien *et al.*, 1992; Sandouk *et al.*, 1993). Chimeric mice derived from *wild type* ES cells and cells with a homozygous deletion of PPARγ showed exclusion of PPARγ null cells from white adipose tissue but not other tissues (Rosen *et al.*, 1999). A role for PPARγ in the induction of genes involved in the accumulation of lipids by adipocytes has been demonstrated (Frohnert *et al.*, 1999; Motojima *et al.*, 1998; Schoonjans *et al.*, 1996; Schoonjans *et al.*, 1995; Tontonoz *et al.*, 1994a).

1.6.3 PPARs indirectly influence TGH mRNA expression

Because the action of TGH generates fatty acids from TG, and the mouse TGH promoter has a PPAR-like response element, an investigation into whether PPARs are involved in TGH expression was performed using PPAR agonists (Dolinsky *et al.*, 2003). PPARγ is expressed in only trace amounts in the fibroblastic pre-adipocyte cell line 3T3-L1. However, during differentiation PPARγ expression precedes the expression of late markers of differentiation, including the adipocyte fatty acid-binding protein aP2 and the transcription factor C/EBPα (Tontonoz *et al.*, 1994a; Tontonoz *et al.*, 1994b). TZDs promote the conversion of pre-adipocytes into adipocytes (Chawla *et al.*, 1994; Kletzien *et al.*, 1992; Sandouk *et al.*, 1993). Since TGH expression begins at a late stage of 3T3-L1 adipocyte differentiation, PPARγ is potentially involved in the induction of TGH expression in mature 3T3-L1 adipocytes. This possibility was explored using PPAR agonists that exhibit selectivity over the other receptor isoforms. The data

indicate that PPARα and PPARγ agonists can increase TGH expression, but the changes reflect secondary responses, and PPARs are not directly involved in TGH expression (Dolinsky *et al.*, 2003). This suggests that TGH expression is not directly regulated by PPARs or fatty acids both in the liver and adipose tissue.

1.7 Conclusions and future perspectives

The characterization of TGH is a major advance since relatively little was known regarding the enzymes that catalyze the lipolysis of stored TG in tissues that do not express HSL or ATGL (the only other identified intracellular TG lipases at neutral pH). Collective data demonstrate that TGH is a lipase and it hydrolyzes stored TG in the hepatocyte. The proposed model (Figure 1-12) is consistent with the intracellular location of TGH and the inability of lipases in other cellular compartments of hepatic cells to facilitate the transfer of stored lipids to VLDL particles. The role of TGH in other tissues requires further investigation, though preliminary data suggests that TGH hydrolyzes stored TG within the adipocyte in a non-hormone stimulated manner.

1.8 Thesis objectives

In hepatocytes, stored TG is mobilized for VLDL assembly through lipolysis, followed by re-esterification. Several lines of evidence indicate that TGH is involved in the lipolysis step, and direct the products of lipolysis towards VLDL assembly. Relatively little is known about the regulation of this protein's function, its specific subcellular localization or determinants of its localization. The hypothesis has arisen stating that TGH in the liver requires a lipid droplet coat protein to facilitate an interaction with its substrate pool. Similarly, lipid droplet coat proteins may block TGH access, thereby regulating lipolysis, mobilization of stored TG and provision of the products of lipolysis for downstream metabolic requirements. The overall goal of the work described in this Thesis was to characterize more precisely where and how TGH operates.

To accomplish this, the following aims were proposed and are divided into respective Chapters:

In Chapter 3, inhibitors of serine esterases, including a TGH specific compound supplied by GlaxoSmithKline, are used on intact cells of hepatic origin to address TGH-mediated contributions to apoB and TG secretion.

In Chapter 4, sequences present in the C-terminus of TGH are analyzed for roles in the subcellular localization of this enzyme.

In Chapter 5, TGH substrate specificity is addressed with emphasis on apoB containing lipoproteins.

In Chapter 6, post-translational modifications of TGH are analyzed, including glycosylation, phosphorylation and acylation. These modifications may be mediators of folding or enzyme activity.

In Chapter 7, proteomic approaches are used to investigate TGH with respect to protein-protein interactions.

In Chapter 8, a summary, concluding remarks and foreseeable future directions are presented.

Chapter 2:

General methodology

2.1 Chemicals

The organic solvents used in this study were obtained from Fisher Scientific, Ottawa, ON, Canada.

4-Methylumbelliferyl heptanoate (MUH), *p*-nitrophenyl esters, Ponceau S protein stain, and bovine serum albumin (BSA; both protease free for immunoblots and essentially fatty acid free for lipid delivery and analysis), were from Sigma-Aldrich, Oakville, ON, Canada.

Nitrocellulose membranes, SDS-PAGE reagents and pre-stained protein molecular mass markers were purchased from Bio-Rad Laboratories, Mississauga, ON, Canada. Secondary antibodies conjugated to horseradish peroxidase were obtained from Pierce Biotechnology, Rockford, IL.

2.2 Molecular Biology Reagents

Standard molecular biology techniques were used for DNA transformation of bacteria, isolation of plasmid DNA, endonuclease digestion of plasmid DNA, ligation of plasmid constructs and polymerase chain reaction (PCR) (Ausubel, 1989; Sambrook *et al.*, 1989).

Oligonucleotides were synthesized by the Institute for Biomolecular Design at the University of Alberta. DNA sequencing was performed by the Molecular Biology Services Unit at the University of Alberta. The plasmid pCI-neo was purchased from Promega, Madison, WI. QuikChange Site-Directed Mutagenesis Kits were from Stratagene, La Jolla, CA. Mutagenesis was also performed by PCR using the strategy outlined in the Stratagene protocol, while substituting *Pwo* DNA polymerase (Roche Applied Science, Indianapolis, IN). Restriction endonucleases and T4-DNA ligase for subcloning of cDNAs were from New England Biolabs, Mississauga, ON, Canada or Boehringer Mannheim, Laval, QC, Canada. All PCRs were performed using a PTC-200, Peltier Thermal Cycler (MJ Research, Inc., Scarborough, ON, Canada). Purification of PCR products for sequencing or cloning into a plasmid was performed using the QIAEX II Gel Extraction Kit from Qiagen (Mississauga, ON, Canada). Plasmid

preparation from bacteria was performed using either the Plasmid QIAprep Spin Miniprep Kit or the HiSpeed Plasmid Purification MIDI kit, both from Qiagen. Remaining molecular biology reagents were from Invitrogen Canada (Burlington, ON, Canada),

2.3 Protein electrophoresis and immunoblot analysis

In preparation for immunoblotting of proteins, tissues and cell culture lysates were processed as described in individual Chapters. Protein concentrations were determined using the Bio-Rad Protein Assay reagent according to the manufacturer's instructions and BSA as a standard. Protein in the samples were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmeli (Laemmli, 1970) and transferred onto nitrocellulose membranes by electroblotting. Membranes were typically blocked 1 to 16 h with 4% skimmed milk powder in Tris buffered saline (TBS; 20 mM Tris pH 7.4, 137 mM NaCl) containing 0.1% Tween 20 (T-TBS), washed several times with T-TBS, and incubated for 1 h at room temperature or 16 h at 4°C with primary antiserum or purified antibodies. These antibodies were diluted in T-TBS containing 4% skimmed milk powder or BSA (essentially protease free) at ratios predetermined to be appropriate for that antibody preparation. Following this incubation, membranes were washed again with T-TBS and incubated with appropriate horseradish peroxidase-conjugated secondary antiserum for 1 h. Typically secondary antibodies were diluted in T-TBS at rations of 1:5,000 or 1:10,000 antibody to T-TBS. Membranes were extensively washed with T-TBS before chemiluminescent detection using the enhanced chemiluminescence detection system (Amersham Corp., Baie d'Urfé, QC). Proteins on the nitrocellulose membranes were routinely stained with Ponceau S by incubating the membrane in the stain for ~1 min, then rinsing the excess stain off with water. This Thesis focuses on the enzyme triacylglycerol hydrolase (TGH), which was routinely probed for by immunoblot using antibodies generated from the purified porcine enzyme (Lehner and Verger, 1997) or from recombinant human TGH produced in bacteria (Alam *et al.*, 2002a). Typically the

anti-porcine TGH antibody was employed at a dilution of 1:1,000 and the secondary antibody (goat anti-rabbit IgG conjugated to horse radish peroxidase) at a dilution of 1:10,000. The anti-human TGH antibody was used at 1:8,000 with the secondary antibody at 1:10,000.

2.4 Silver staining of polyacrylamide gels

Following electrophoresis, gels were soaked in destaining solution containing water/methanol/acetic acid 50:40:10 (by volume) for a minimum of 1 h. The gels were rinsed twice for 15 min in water, then in 0.2 g/L sodium thiosulfate (sensitization solution) for approximately 1 min. The gels were rinsed again with water as above, then incubated for 20-30 min in solution containing 0.2% silver nitrate and 1 mM formaldehyde (silver staining solution). After another set of rinses with water, gels were soaked in solution containing 6% sodium carbonate, 6 mM formaldehyde and 20 μM sodium thiosulfate (developing solution) until the protein in the gels became visible and achieved a desirable level of stain. The staining reaction was halted by incubation in the destaining solution defined above.

2.5 Cell culture

All cell culture and incubations were performed at 37°C in humidified air (89-91% saturation) enriched with 5% CO_2 and in the presence of 100 units/mL penicillin and 100 μg/mL streptomycin unless specifically indicated otherwise. Individual cell lines were maintained as described below and manipulated as indicated in individual Chapters. Antibiotics, Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and horse serum were purchased from Invitrogen Canada (formerly Gibco-BRL Life Technologies) Burlington, ON, Canada.

2.5.1 Maintenance of cell lines

McArdle RH7777 cells were obtained from American Type Culture Collection (ATCC; Manassas, VA) and cultured in DMEM containing 10% (v/v)

FBS and 10% horse serum. McArdle RH7777 cells are a rat hepatoma cell line. They were well suited for the studies in this Thesis as they can secrete lipids on particles of clinical relevance (VLDL) (Lehner and Vance, 1999). The lipidation state of these particles can be affected by transfection of the TGH cDNA (Lehner and Vance, 1999).

Cos-7 cells (from ATCC) were cultured in DMEM containing 10% FBS. Sf-9 cells were cultured at 28°C with GIBCO BRL Sf-900 II supplemented with 5% FBS, 50 U/mL penicillin and 50 μg/mL streptomycin. HepG2 cells (from ATCC) were cultured in Minimum Essential Medium (MEM) containing 0.1 mM nonessential amino acids, 1 mM sodium pyruvate and 10% FBS.

2.5.2 Preparation of primary hepatocytes

Rat or mouse hepatocytes were isolated from male animals fed *ad libitum* by collagenase perfusion of the liver. Hepatocytes were isolated from male Sprague-Dawley rats (body weight 100-175 g) or male C57BL/6J mice by collagenase perfusion of the livers. All animals were fed *ad libitum* until sacrifice.

The preparation of hepatocytes was performed based on previously published procedures (Yao and Vance, 1988). The rat or mouse was anesthetized (using methoxyflurane), and then the abdominal cavity was opened by making a midline incision through the skin and abdominal muscle. Sodium pentobarbital (approximately 100 μL) was often injected under the skin where the incision would be made at the discretion of the handler. The intestines were moved out of the abdominal cavity to expose the hepatic portal vein. A ligature (thread) was set in place loosely around the hepatic portal vein to close the liver when required. As well, the lower vena cava was loosely tied above the renal vein. The portal vein was clamped as far away from the liver as possible. The vein between the clamp and ligature was cut and an 18-gauge needle inserted into the portal vein. The needle was tied in position with the prepared ligature. To perfuse the liver, a peristaltic pump with cannula tubing to the needle was used at a speed of 4 mL/min to deliver the solutions described below.

***Perfusing Solution:**

Hanks Balance Salt Solution (Gibco 14185)

20 mM glucose

4.0 mM $NaHCO_3$

2.5 mM HEPES pH 7.4

0.5 mM EGTA

Use at 37 °C

The vena cava was promptly severed below the ligature. Typically, all lobes of the liver cleared within a few seconds. The pump speed was increased after about 15 sec to 5 mL/min, and perfusion continued for 3-5 min or until the liver was visually assessed to be clear of blood. While the perfusate was flowing, the abdominal muscle flap was removed by cutting through the left side of the rib cage and the diaphragm. The upper vena cava was tied loosely where it was visible below the heart. The lower vena cava was tied off with the ligature put in place previously, and the atrium cut. The perfusate was allowed to run out of the heart until no blood was obvious to the eye. The upper vena cava was tied off and the liver allowed to swell. Perfusion was terminated when the liver looked clear (3-5 min after tying off the vena cava).

The solution pumping through the liver was changed to collagenase containing solution (see below). Perfusion with this solution was performed for 6-10 min. Degradation of the liver was estimated by "poking" with forceps as the tissue gets soft during perfusion. The procedure was continued when the liver was soft, and integrity was compromised as assessed with the forceps.

***Collagenase solution:**

Hanks Balance Salt Solution (Gibco 14065)

20 mM glucose

4.0 mM $NaHCO_3$

2.5 mM HEPES pH 7.4

100 U/mL collagenase (Sigma C-0130)

Use at 37 °C

The liver was removed from the animal and transferred to a 100 mm culture dish for disruption with scissors. The "mash" was transferred to a 50 mL conical tube containing 35 mL of the collagenase containing solution and placed in a shaking 37°C water bath for ~1 min or until "lumps" were no longer readily observed. Further disruption of the tissue was accomplished by pipetting cells up and down for about 3 min. The cells were filtered through a sterilized (autoclaved) metal sieve into a 50 mL conical tube. The cells were spun down at 500 x*g* in a swinging bucket rotor at room temperature for 2 min. The supernatant was removed by aspirating it off, then 20 mL of DMEM containing 15% FBS were added and cells were resuspend by pipetting up and down several times. The cells were filtered again through another sterilized sieve into a 50 mL conical tube. The cells were spun down as above and then resuspended in 20 mL DMEM containing 15% FBS using a pipette. The procedures result in removal of collagenase and undigested fragments of tissue. An aliquot of the cells were counted using a hemocytometer and 2 million cells were plated in 60 mm collagen coated dishes (total volume of 2 mL) or 5 million cells in 100 mm collagen coated dishes or flasks (total volume of 7 mL).

2.6 Stable transfection of McArdle RH7777 cells

Construction and preparation of plasmids for transfection is described in the relevant Chapters. Stable transfections began by plating $1.6x10^6$ McArdle RH7777 cells in a 60 mm diameter dish and growing them overnight. Three μg of plasmid were introduced using Lipofectamine2000 according to the manufacturer's instructions. Briefly, 3 μg of plasmid DNA in 10 mM Tris pH 8.5 was diluted to 200 μL with DMEM. For each transfection, 8 μL of Lipofectamine2000 was also diluted to 200 μL with DMEM. These two solutions were combined and allowed to stand at room temperature for 20 min. The cells were rinsed with PBS, then 1.6 mL of DMEM containing 10 % FBS and 10% HS were added. The plasmid containing mixture was introduced by gently pipetting it into this media, bringing the total volume to 2 mL. Cells were grown for 24 h

after transfection, then passaged 1:10 into 100 mm dishes and DMEM containing 1.6 mg/mL of Geneticin sulfate and sera. After 2-3 days, cell death induced by Geneticin became readily apparent. Cells remaining adherent were washed with PBS to further remove dead cells and debris; media were replaced with DMEM containing sera and 1.6 mg/mL of Geneticin. After ~1 week with regular media changes, single colonies became obvious on the culture dishes. Single colonies were selected and characterized. Stably transfected cell lines were maintained in growth medium containing 0.4 mg/mL of Geneticin.

2.7 Augmentation of intracellular triacylglycerol

In cell culture systems, TG stores were augmented by incubating cells with 0.4 mM oleic acid complexed to 0.5% (w/v) BSA (essentially fatty acid free). A 20x BSA/oleate stock was prepared by dissolving 5 g BSA in 50 mL DMEM by stirring (~5 min). This was warmed to 56°C in a water bath. Meanwhile, 0.106 g of oleate (an oil) was weighed out in a 100 mL beaker, then warmed by holding the beaker in the 56°C bath for ~1 min. The BSA/DMEM mixture was added to the beaker containing the warm oleate and stirred vigorously with a Teflon coated stir bar until the solution clarified (~2 min). This solution was sterile filtered through a 0.22 µm vacuum assisted filter while it was still warm. After incubating cells in media containing a 1x amount of the stock fatty acid solution (at least 4 h), cells were washed three times for 10 min with DMEM containing 0.5% BSA to remove labeled fatty acids that were non-specifically associated with the exterior of the cells.

2.8 Lipid extraction and analyses

Lipids were extracted from samples with chloroform/methanol (2:1, v/v) (Folch *et al.*, 1957) containing 100 µg each of non-radioactive lipid carriers (phosphatidylcholine (PC), oleic acid, trioleoylglycerol and cholesteryl oleate) when radio-labeled cellular lipids were to be analyzed (Lehner and Vance, 1999). These lipid carriers facilitate visualization of the lipids after separation by thin-layer chromatography (see below). Typically 4 mL of the chloroform/methanol

based cocktail were used for extraction of 1 mL of cell homogenate or culture media. The chloroform phase (containing the lipids) was isolated, dried under nitrogen, re-dissolved in a small volume of chloroform and applied to silica gel H thin layer chromatography plates (Whatmann Inc., Florham Park, NJ). The plates were developed to 1/3 the height with chloroform/methanol/acetic acid/water 25:15:4:2 (v/v) to allow separation of glycerophospholipids, followed by development in heptane/isopropyl ether/acetic acid 60:40:4 (v/v) to separate neutral lipids (cholesterol fatty acids, TG and cholesteryl esters (CE)) (Lehner and Vance, 1999). When analyzing the neutral lipids only, or when analyzing total glycerolipids, only the latter solvent system was employed. The lipid classes were visualized by exposure of TLC plates to iodine vapour and were identified by comparing their migration with standards. Bands were scraped off the plates and the lipids dissolved in 5 mL of CytoScint scintillation fluid (ICN Biomedicals, Irvine, CA). The radioactivity associated with each lipid was determined by scintillation counting on a Beckman LS 6000TA apparatus.

2.9 In vitro lipase assays

Lipase activity was assayed using two methods. The first was a fluorescence based assay performed using the fluorogenic substrate, 4-methylumbelliferyl heptanoate (MUH) as described (Dolinsky *et al.*, 2004a; Gilham *et al.*, 2005). A 100 mM stock of MUH was prepared in tetrahydrofuran, then diluted to 1 mM MUH in 20 mM Tris buffer, pH 8.0, 1 mM EDTA, 300 μM taurodeoxycholate immediately before initiating the reaction. The sample to be tested (often 10 μg of protein from a cell homogenate or 5 μg of protein from a microsomal preparation) was introduced into a 96 well black microtitre plate and brought to 180 μL using the same buffer used to dilute the substrate above. The enzymatic reaction was initiated with the injection of 20 μL of the 1 mM MUH into the plate (total volume 200 μL; final substrate concentration 100 μM). The plate was agitated at room temperature and read with a Fluoroskan Ascent FL Type 374 (Thermo Labsystems) in a kinetic fashion for up to 5 min (excitation/emission wavelengths: 355/460 nm). Data were analyzed using Ascent software version

2.4.2. The amount of substrate consumed was quantitated using fluorescence values generated with a standard solution of 4-methylumbelliferone (sodium salt).

For the second assay, lipase activity was monitored spectrophotometrically at 405 nm via the liberation of *p*-nitrophenol from *p*-nitrophenyl esters including laurate, palmitate and acetate as previously described (Alam *et al.*, 2002b; Lehner and Vance, 1999; Lehner and Verger, 1997). Briefly, protein samples were incubated in a 0.2 mL assay solution containing 50 mM Tris (pH 8.0), 150 mM NaCl, 0.01% Triton X-100 and 250 μM *p*-nitrophenyl ester substrate. After an incubation period, the release of *p*-nitrophenol (PNP) was measured in 96 well clear microtitre plates as the absorbance at 405 nm on a Molecular Devices SpectraMax 250 spectrophotometer.

Chapter 3:

The impact of inhibition of TGH on VLDL secretion

The majority of the information presented in this Chapter has been published in Gilham, D., S. Ho, M. Rasouli, P. Martres, D.E. Vance, and R. Lehner. (2003) Inhibitors of hepatic microsomal triacylglycerol hydrolase decrease very low density lipoprotein secretion. *FASEB J.* 17:1685-1687.

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3.1 Introduction

3.1.1 Plasma lipid and human disease

The metabolic syndrome is a term describing a combination of insulin resistance, hypertension, and visceral obesity that often occur together (reviewed in (Grundy, 1997; Grundy, 1998)). Hypertriglyceridemia caused by increased plasma concentrations of apoB is the most consistent lipid disorder in the metabolic syndrome as well as a risk factor for cardiovascular disease (Grundy, 1997; Hodis and Mack, 1995). Although the precise regulation of apoB metabolism has not been fully elucidated, the underlying mechanism may be related to oversecretion or impaired clearance of TG-rich lipoproteins, or both.

Elevated VLDL and apoB in plasma represent independent risk factors for cardiovascular disease (NCEP, 2001). The liver assembles and secretes apoB containing lipoproteins, mainly as VLDL. Hepatic VLDL assembly is an intricate process that is largely regulated by the provision of lipid to maturing primordial apoB containing particles (Borchardt and Davis, 1987; Dixon *et al.*, 1991; White *et al.*, 1992). Inadequate lipidation of the primordial particles results in intracellular degradation rather than secretion (reviewed in (Fisher and Ginsberg, 2002; Yao *et al.*, 1997)). The major lipid component of VLDL particles is TG (Davis and Vance, 1996). Hence mobilization of stored TG represents a potentially regulated step in VLDL assembly and secretion.

3.1.2 Mobilization of TG from storage pools to VLDL assembly

In hepatocytes, TG is stored in neutral lipid droplets (see Section 1.23). The majority (as much as 70%) of VLDL associated TG is derived from storage pools rather than *de novo* synthesis (see Section 1.11). Lipolysis of stored TG to DG/MG followed by re-esterification to TG is required prior to incorporation into an apoB containing particle. Until recently, intracellular enzymes involved in the hydrolysis of stored TG have not been determined with the exception of hormone sensitive lipase (HSL), which mainly functions to mobilize stored TG in adipose tissue (Strålfors *et al.*, 1987)). HSL is not found in appreciable quantities in the liver. In addition, when HSL was ectopically expressed in HepG2 cells, the fatty

acids released by the enzyme were diverted into the oxidative, rather than into the secretory pathway, suggesting that subcellular localization of the lipase may be important in the channeling of substrates (Pease *et al.*, 1999).

The enzyme triacylglycerol hydrolase (TGH), initially purified from porcine liver microsomes, has been suggested to play a role in the lipolysis that occurs when TG is mobilized for VLDL assembly (Lehner and Verger, 1997). TGH is a soluble protein that localizes to the lumen of ER (see Chapter 4) and is also found in association with lipid droplets isolated by subcellular fractionation (Lehner *et al.*, 1999). Hepatoma cell lines lacking this enzyme (e.g. McArdle RH7777 cells and HepG2) are inefficient in the mobilization of stored TG for VLDL secretion (Gibbons *et al.*, 1994; Lehner and Vance, 1999; Wu *et al.*, 1996). Both of these hepatoma cell lines are capable of secreting lipid poor apoB containing particles of non-physiologically high density. Upon the withdrawal of an extracellular supply of fatty acid, McArdle RH7777 cells can secrete small amounts of apoB of VLDL density. It has been suggested that the impaired secretion of VLDL from TG-laden HepG2 cells is due to a defect in the mobilization of TG from storage pools (Gibbons *et al.*, 1994; Wu *et al.*, 1996). It was demonstrated that exogenous oleate included in incubations with HepG2 cells entered a "secretion-coupled" pool in microsomes that stimulated apoB secretion with newly made TG, rather than utilizing TG from storage pools (Wu *et al.*, 1996). When oleate was withdrawn from the medium, stored TG was not available for secretion. Data from our laboratory and contained in this Thesis support the concept that this defect, in part, is due to a lack of TGH expression, as this enzyme is responsible for mobilizing TG from storage depots. The rat TGH cDNA had been previously cloned in our laboratory and used to stably transfect McArdle RH7777 cells (Lehner and Vance, 1999). TGH-transfected cells had a higher capacity to hydrolyze stored TG and direct lipolytic products into the secretory pathway, resulting in increased lipidation of secreted apoB, particularly apoB100 (Lehner and Vance, 1999).

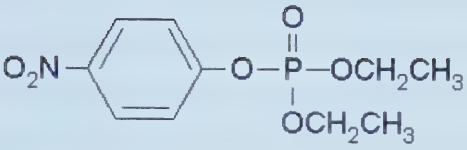
3.1.3 Chapter objectives

To test the hypothesis that TGH plays a role in the provision of core lipids for the assembly of VLDL, lipase activity was inhibited in hepatoma cells stably transfected with the TGH cDNA and in primary rat hepatocytes followed by analysis of lipid and apoB synthesis and secretion. The cell permeable inhibitor compounds included the non-specific lipase/esterase inhibitor diethyl-*p*-nitrophenyl phosphate (E600), and a specific TGH inhibitor, 4,4,4-trifluoro-2-[2-(3-methylphenyl)hydrazono]-1-(2-thienyl)butane-1,3-dione (GR148672X). These studies provide the basis for development of pharmacological agents that target TGH in order to decrease the amount of VLDL secreted from the liver and, as a consequence, decrease the risk of cardiovascular disease and stroke related to atherosclerosis.

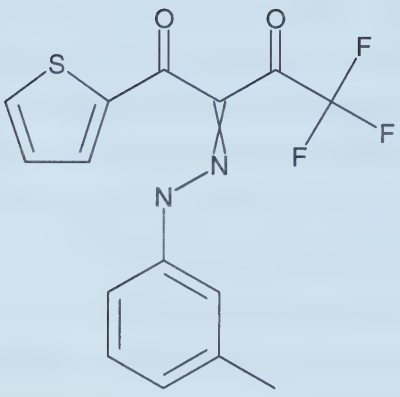
3.2 Materials and Methods

3.2.1 Reagents

Oleic acid, trioleoylglycerol, essentially fatty acid-free BSA, *p*-nitrophenyl fatty acyl esters, protein A-Sepharose CL 4B, diethyl-*p*-nitrophenyl phosphate (E600), fumed silica (Cab-O-Sil), Sigma Diagnostics Infinity Triglyceride Reagent and anti-sheep IgG conjugated to horseradish peroxidase were purchased from Sigma-Aldrich (Oakville, ON, Canada). [1(3)-^{3}H] glycerol (2.6 Ci/mmol), [^{14}C] glycerol (149 mCi/mmol), [9,10(n)-^{3}H] oleic acid (10 Ci/mmol), L-[4,5-^{3}H] leucine (57 Ci/mmol) were obtained from Amersham Canada (Oakville, ON, Canada). EasyTag Expre^{35}S^{35}S protein labeling mix (11.23 mCi/mL) was obtained from PerkinElmer (Boston, MA). Minimum Essential Medium (MEM), DMEM and sodium pyruvate were from Gibco BRL (Life Technologies Inc., Grand Island, NY). Sheep anti-human polyclonal apoB antibody was from Boehringer-Mannheim (Laval, QC, Canada). All other chemicals and solvents were of reagent or better quality and were obtained from local suppliers. GR148672X, a specific inhibitor of TGH, was developed in collaboration with GlaxoSmithKline (Les Ulis, France). The chemical structures of E600 and GR148672X are shown in Figure 3-1.



diethyl-*p*-nitrophenyl
phosphate
(E600)



GR148672X
(GSKi)

Figure 3-1 Chemical structures of lipase inhibitor compounds. E600 is a non-specific inhibitor of serine esterases, while GR148672X is a TGH specific compound developed in collaboration with GlaxoSmithKline. Paul Martres is a chemist with GlaxoSmithKline central in the development of GR148672X.

3.2.2 Subcellular fractionations

Microsomal membranes from male 225-250g Sprague-Dawley rat livers were prepared essentially as previously described (Lehner *et al.*, 1999). Briefly, rats were exsanguinated via the abdominal aorta; livers were removed and washed with ice-cold TBS. Twenty percent liver homogenates were obtained using a Waring blender for 30 sec at low speed in 250 mM sucrose, 50 mM Tris, pH 7.4, and 5 mM EDTA. Unbroken cells and tissue debris were removed by centrifugation for 10 min at 500 x*g*. Crude mitochondrial pellets were obtained by centrifugation of the 500 x*g* supernatant at 15,000 x*g* for 10 min and microsomal membranes were prepared by centrifugation of the 15,000 x*g* supernatant for 1 h at 106,000 x*g*. Microsomes from McArdle RH7777 cells were prepared similarly except that sonication was used for cell disruption (Lehner *et al.*, 1999; Lehner and Vance, 1999).

3.2.3 Purification of rat liver TGH

Rat liver TGH was purified from detergent-solubilized microsomes by a procedure described previously for the purification of TGH from porcine liver (Lehner and Verger, 1997). The identity of TGH was confirmed by N-terminal protein sequencing performed by the Alberta Peptide Institute at the University of Alberta.

3.2.4 Lipase assay

Lipolytic activities in microsomal membranes prepared from rat liver, empty vector transfected (stable) and rat TGH cDNA-transfected McArdle RH7777 cells were assessed using the chromogenic substrates *p*-nitrophenyl laurate or palmitate as outlined in Chapter 2 (Section 2.9).

3.2.5 Inhibition of TGH

Purified rat TGH or microsomes were preincubated for 30 min in the presence or absence of 100 μM E600 or 10 μM GR158672X on ice prior to

addition of the lipase substrate. The effectiveness of the inhibitor in intact cells was assessed by incubation for 2 h in DMEM with or without the inhibitors. The cells were washed, microsomes were prepared and microsomal lipase activity was determined using *p*-nitrophenyl laurate as described in Section 2.9.

3.2.6 Secretion of lipid stores from hepatoma cells

McArdle RH7777 cells (CMV vector-only and TGH-transfected) (Lehner and Vance, 1999) and HepG2 cells at 70% confluency in 60 mm dishes were incubated with 2 mL of 0.4 mM oleic acid/0.5% BSA in DMEM and 10 μCi/dish [^{3}H] glycerol for 12 h. Cells were washed and incubated 2 h with 100 μM E600 or acetonitrile (control cells). The final concentration of acetonitrile was 0.1%. Cells were then incubated for 6 h in 2 mL DMEM ± E600 (100 μM). Media were collected, centrifuged and 1 mL used for lipid analysis (below). Cells, were washed with ice-cold PBS, harvested in 2 mL PBS, sonicated and analyzed for protein content. The lipids were then analyzed as described below.

3.2.7 Turnover and secretion of lipids in primary rat hepatocytes

Hepatocytes plated on collagen-coated 60 mm dishes at 2.5 x10^6 cells/dish were incubated in DMEM containing 15% FBS for 6 h. Cells were then washed with DMEM and incubated for 12 h in DMEM without sera. Cells were then incubated for 4 h in 2 mL DMEM containing 0.4 mM [^{3}H] oleic acid (21,000 dpm/nmol) complexed to 0.5% fatty acid free BSA and 1.5 x10^6 dpm/dish [^{14}C] glycerol in the presence or absence of E600 (100 μM) or GR148672X (10 μM). Following the incubation, some cells and media were collected for analysis (pulse). Remaining dishes that were incubated in the absence of an inhibitor were washed with DMEM and incubated 1 h with DMEM to allow secretion of lipoproteins containing *de novo* synthesized lipids. Cells were then incubated with DMEM ± the respective lipase inhibitors for 1 h, a time determined to be sufficient for the compounds to enter the cell and inhibit microsomal TGH. The media were discarded, replaced with fresh media ± E600 (100 μM) or

GR148672X (10 μM) and incubated for an additional 4 h. Finally, cells and media were collected for analysis (chase).

Cellular and secreted TG mass was determined with Sigma Diagnostic Infinity TG Reagent in a colourimetric assay. Briefly, lipids from cells and media following the chase period were extracted with chloroform/methanol (2:1, v/v), dried under nitrogen and resuspended in 20 μL of ethanol. One mL of the TG reagent was added and TG content was determined spectrophotometrically at 520 nm according to the instructions from the manufacturer. Trioleoylglycerol was used as a standard.

3.2.8 Lipid analysis

Lipids were extracted from both cells and media with organic solvent, separated by thin layer chromatography and the radioactivity associated with each lipid species determined by scintillation counting using the procedures described in Section 2.8.

3.2.9 Secretion of apoB by primary rat hepatocytes

Primary rat hepatocytes in 60 mm dishes were washed and incubated 4 h with DMEM containing 0.4 mM oleic acid/0.5% BSA. Cells were then washed and incubated 2 h with 2 mL of DMEM ± E600 (100 μM) or GR148672X (10 μM) followed by a wash with DMEM without leucine ± E600 (100 μM) or GR148672X (10 μM) ± lactacystin (10 μM). Lactacystin is an inhibitor of proteosome-mediated protein degradation. Cells were then incubated 2 h with 2 mL DMEM without leucine and 250 μCi of [^{3}H] leucine ± E600 (100 μM) or GR148672X (10 μM) ± lactacystin. Media were removed and briefly centrifuged (2,600 x*g* for 2 min) to remove cellular debris. Cells were collected in immunoprecipitation buffer (Lehner and Vance, 1999) (150 mM NaCl, 50 mM Tris pH 7.4, 5 mM EDTA, 0.5 % Triton X-100, and 0.1% SDS) containing 1 mM benzamidine, homogenized by sonication. Protein concentrations in the lysates were determined as described in Section 2.3 using Bio-Rad protein assay reagent. ApoB immunoprecipitation and analysis was performed as previously described (Lehner and Vance, 1999).

Briefly, 1 mL of cell sonicates or culture media (adjusted with 10x immunoprecipitation buffer and containing 1 mM final concentration of benzamidine) was combined with 10 µL of anti-human apoB IgG. The mixture was incubated on a rotating rack for 12 h at 4°C, then 50 µL of protein A Sepharose was added and incubation continued for 3 h. The beads were pelleted by brief centrifugation, washed 3 times with an excess of immunoprecipitation buffer, then 0.1 µg of rat VLDL protein was added, followed by denaturing electrophoresis sample buffer. Samples were boiled and separated by 5% polyacrylamide gel electrophoresis in 0.1% SDS. Gels were silver stained using the protocol given in Section 2.4. Bands corresponding to apoB48 and apoB100 were excised, dissolved at 60°C in 0.2 mL of 60% perchloric acid followed by 0.4 mL of 30% hydrogen peroxide and the radioactivity associated with apoB48 and apoB100 was determined using Hionic-Fluor scintillation cocktail (Packard Instrument Co., Meriden, CT) and scintillation counting on a Beckman LS 6000TA instrument.

The mass of apoB in media following a 4 h chase was also analyzed. Fumed silica (Cab-O-Sil) was added to the media (3 mg silica/mL media) and the mixture was incubated for 45 min at 4°C. ApoB bound to silica was collected by brief centrifugation (1 min at 5,000 rpm), the pellet was washed, resuspended in denaturing electrophoresis sample buffer, boiled and proteins were resolved via SDS-PAGE (5%). Proteins were transferred to a nitrocellulose membrane and probed with anti-human apoB IgG (1:5,000 dilution) followed by a secondary antibody (donkey anti-sheep IgG-HRP 1:5,000 dilution). Reactivity was detected by enhanced chemiluminescence and the relative intensity of the resulting bands on the blot was analyzed by densitometry using Bio-Rad Quantity One software.

3.2.10 Pulse-chase analysis of apoB synthesis in primary rat hepatocytes

Primary rat hepatocytes in 60 mm dishes were washed and incubated overnight with DMEM containing 15% FBS and 0.4 mM oleic acid plus 0.5% BSA. Cells were then washed and incubated for 1 h with 2 mL of methionine- and cysteine-free DMEM with or without E600 (100 µM) or GR148672X (10 µM).

Cells were then incubated in methionine- and cysteine-free DMEM with or without E600 (100 μM) or GR148672X (10 μM) containing 50 μCi/mL [^{35}S] labeled methionine and cysteine for 15 min. Cells were washed and incubated in 2 mL of DMEM (with methionine and cysteine) ± lipase inhibitors for 30 min. Media were collected and cleared of cellular debris by centrifuging at 2,600 x*g* for 2 min. Cells were scraped into 2 mL of immunoprecipitation buffer containing a protease inhibitor cocktail (Roche Diagnostics) and were briefly sonicated. Aliquots (1 mL) of media and sonicates were precleared via a 3 h incubation at 4°C with 20 μL of protein A-Sepharose beads. The beads were sedimented by centrifugation for 2 min at 1,000 x*g*. Immunoprecipitations of apoB from the supernatants, SDS-PAGE, and transfers to nitrocellulose membranes were performed as described above. Quantitation of newly synthesized apoB that had incorporated radio-labeled amino acids was accomplished using a PhosphorImager. Resulting bands were quantified as above using Bio-Rad Quantity One software.

3.2.11 Lactate dehydrogenase (LDH) assay

Primary rat hepatocytes in 60 mm dishes were washed and incubated overnight with DMEM containing 15% FBS and 0.4 mM oleic acid/0.5% BSA. Cells were then washed and incubated for 1 h with 2 mL of DMEM with or without E600 (100 μM) or GR148672X (10 μM). Media were removed, and cells were re-introduced into 2 mL of DMEM with or without E600 or GR148672X and incubated for 4 h. Some cells were incubated for 1 h with 2 mL of DMEM containing 400 μM *tert*-butyl hydroperoxide. This treatment results in disruption of cell integrity and release of the cytosolic LDH into the medium. Samples of medium were collected after treatments and cleared of cellular debris by centrifugation at 2,600 x*g* for 2 min. Cells were scraped into 1 mL of PBS containing a protease inhibitor cocktail (Roche Diagnostics) and were briefly sonicated. LDH activity in cells and media was determined as described previously (Haidara *et al.*, 2002; Moldeus *et al.*, 1978). Assays were conducted in triplicate with 15 μL of culture medium or 7.5 μL of cell sonicates

supplemented with 7.5 µL of PBS. The assay was started by addition of 250 µL of substrate solution containing 100 mM phosphate buffer, pH 7.4, 1.4 mM sodium pyruvate, and 0.2 mM NADH. Absorbance at 340 nm was then monitored every minute for 10 min. LDH activity is expressed as the percentage of media LDH activity relative to total (cells plus media) activity after 3 min of reaction time.

3.2.12 In vivo inhibition of TGH in hamsters

Hamsters fed *ad libitum* were treated once per day orally with GR148672X (25 mg/kg) or solvent alone (DMSO) for 3 days. The animals were fasted for four hours following the last administration of the TGH inhibitor, then sacrificed. Plasma lipids as well as apoB protein levels were analyzed.

A single-oral dose of GR148672X (25 mg/kg) or solvent alone (DMSO) was administrated orally to hamsters. Four hours later, plasma lipoproteins and their composition were analyzed by HPLC. The experiments in hamsters were performed by collaborators at GlaxoSmithKline.

3.3 Results

3.3.1 Inhibition of microsomal lipase activity

Similar to porcine hepatic microsomes (Lehner and Verger, 1997), rat hepatic microsomes contain about 70% of total hepatic alkaline lipase activity, with the majority of the microsomal lipase activity co-purifying with TGH. Figure 3-2 shows the inhibition of lipolytic activity by E600 in rat hepatic microsomes and of purified TGH. E600 (100 µM) inhibited more than 90% of the lipase activity in microsomes and more than 95% of the activity using the purified enzyme.

3.3.2 Inhibition of TGH activity in intact cells by E600

The rat hepatoma cell line McArdle RH7777 does not express TGH (Lehner *et al.*, 1999; Lehner and Vance, 1999), hence cell lines stably

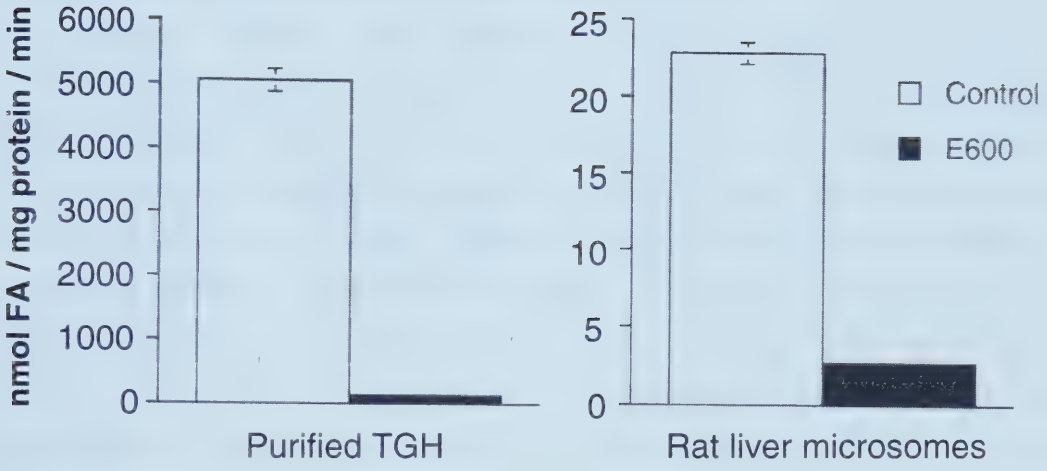


Figure 3-2 Inhibition of purified and microsome-associated TGH by E600. Enzyme preparations were incubated on ice for 30 min ± E600 (100 μM). Lipase activity was then determined using *p*-nitrophenyl laurate as a substrate as described in Materials and Methods. FA: fatty acid. Data represent means ± SD from three independent experiments. This figure was provided by Samuel Ho and Dr. Richard Lehner.

transfected with either pBK-CMV or with pBK-CMV containing the rat TGH cDNA were generated (Lehner and Vance, 1999). Incubation of McArdle RH7777 cells with E600 followed by analysis of remaining microsomal lipolytic activity indicated that E600 entered the cell and the ER, where TGH resides, and inhibited the TGH activity (Figure 3-3). The inhibitor had little effect on the endogenous lipolytic activity present in McArdle RH7777 cell microsomes.

3.3.3 E600 does not affect glycerolipid secretion from wild type McArdle RH7777 and HepG2 cells but inhibits TGH-dependent lipid secretion

McArdle RH7777 cells expressing TGH demonstrated increased mobilization and secretion of stored cellular TG (Lehner and Vance, 1999). *Wild type* McArdle RH7777 and HepG2 cells are deficient in the mobilization of stored TG for lipoprotein assembly and secretion (Gibbons *et al.*, 1994; Lehner and Vance, 1999; Wu *et al.*, 1996). Figure 3-4 shows that the TGH-dependent secretion of stored TG was inhibited by E600. As expected, very little or no effect on secretion of preformed TG was seen in empty vector transfected McArdle RH7777 cells or in HepG2 cells. The inhibitory effect appeared to be TG-specific as phospholipid secretion was not significantly affected by the inhibitor treatment.

3.3.4 Mobilization of lipids in primary rat hepatocytes

To further assess the function of TGH and its role in the mobilization of stored cellular TG for VLDL assembly, inhibition studies were carried out in primary rat hepatocytes where the role of the lipolysis/re-esterification pathway in VLDL formation has been established (Gibbons *et al.*, 2000; Lankester *et al.*, 1998; Wiggins and Gibbons, 1992; Yang *et al.*, 1996). Cellular lipid synthesis, turnover and secretion was followed from the point of the acyl acceptor (glycerol backbone of glycerolipids) and acyl donor (fatty acid) by incubating the cells with [^{14}C] glycerol and [^{3}H] oleic acid (Figures 3-5 (cellular lipids) and 3-6 (secreted lipids)). Following labeling of cellular lipids, cells were incubated for 1 h to allow secretion of lipoproteins containing *de novo* synthesized lipids (wash period)

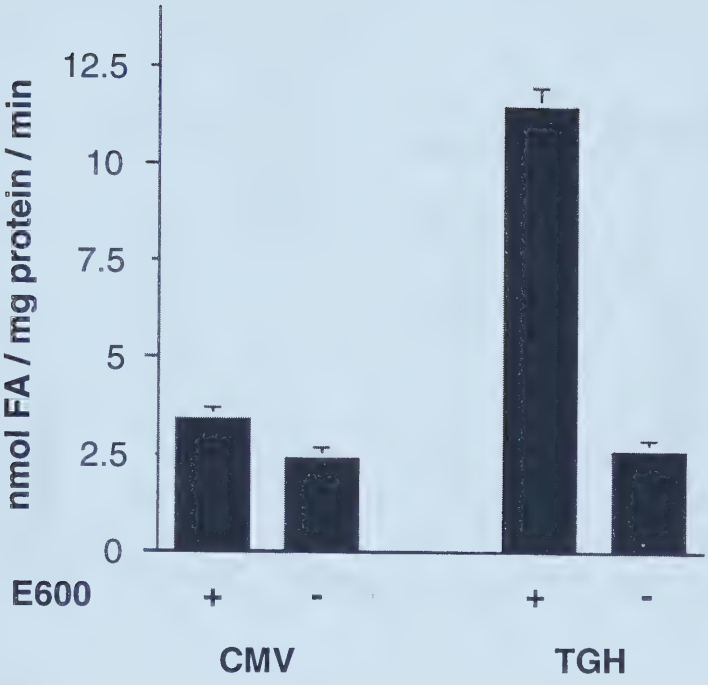


Figure 3-3 Inhibition of TGH by E600 in intact cells. McArdle RH7777 cells stably transfected with an empty vector (CMV) or the rat TGH cDNA (TGH) were incubated 4 h ± E600 (100 μM). Cells were then harvested, disrupted by brief sonication, microsomes were isolated and lipolytic activity was determined *in vitro* using *p*-nitrophenyl palmitate as a substrate as described in Materials and Methods. FA, fatty acid. Data represent means ± SD from three independent experiments. The figure was developed by Dr. Mehdi Rasouli.

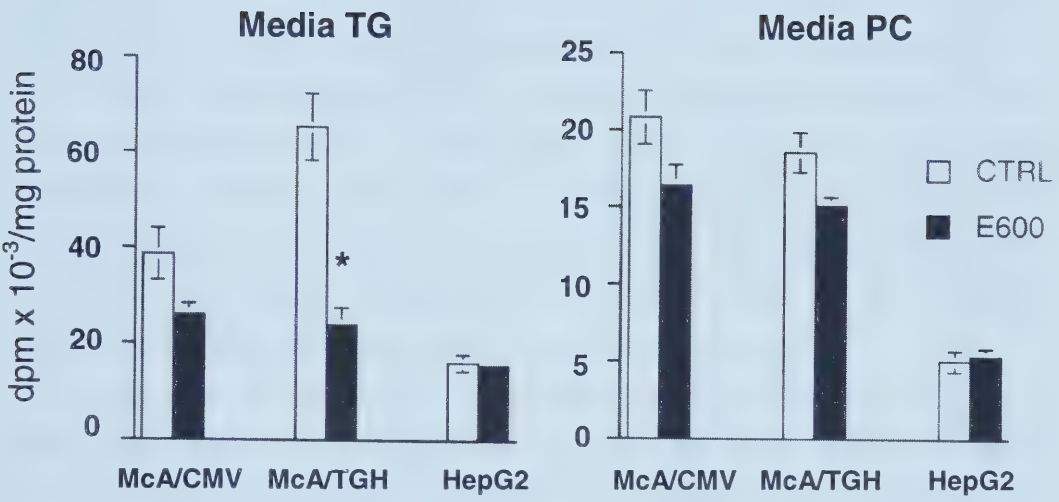


Figure 3-4 Secretion of radio-labeled lipid from hepatoma cells preincubated with or without E600. McArdle RH7777 cells transfected with empty vector (CMV) or the rat TGH cDNA and HepG2 cells were incubated 12 h with 0.4 mM oleic acid in the presence of radio-labeled glycerol to generate labeled glycerolipid pools. Cells were then washed and incubated for 2 h ± E600 (100 μM). Cells were then incubated ± E600 for 6 h. Cells and media were collected and radioactivity in the various lipid classes were determined as described in Materials and Methods. McA, McArdle RH7777. Data represent means ± SD from three independent experiments. *P<0.005. This figure was developed by Samuel Ho and Dr. Richard Lehner.

followed by another 1 h incubation in the presence or absence of E600 (chase period). During the chase period, 57% of cellular glycerol-labeled TG was turned over in the absence of the inhibitor ($P<0.009$) compared to only 19% turnover (not significant) in the presence of the inhibitor (Figure 3-5 A). PC turnover was not affected by E600 (Figure 3-5 B). Secretion of stored glycerol-labeled TG was decreased by 41% ($P<0.015$) in the presence of E600 (Figure 3-6 A). This is further evidence that lipolysis is an important step for secretion of stored TG.

Primary hepatocytes are able to secrete relatively constant levels of TG following removal of oleate from the culture media (Lehner and Vance, 1999) (see below). However, only 18-20% of TG labeled in the glycerol backbone was secreted (Figure 3-6 A), which indicates a dilution of the [^{14}C] glycerol released by lipolysis with unlabeled glycerol before re-esterification. This observation agrees with the previous results (Wiggins and Gibbons, 1992).

Very little (if any) *de novo* fatty acid synthesis occurs in primary rat hepatocytes during 4 h of culture in the presence of exogenous fatty acid because of inhibition of acetyl-CoA carboxylase (Nikawa *et al.*, 1979). Under these conditions, the contribution of *de novo* lipogenesis to VLDL associated TG is minimal, and the exogenous fatty acid is the primary contributor to TG synthesis. Hence the specific radioactivity of the exogenous oleate correlates well with the TG mass. Using radio-labeled oleic acid as the tracer also allows for the investigation of CE metabolism. During the pulse period the incorporation of exogenous radio-labeled fatty acid (21,000 dpm/nmol) into lipids was as follows: 94 nmol into TG (Figure 3-5 C, pulse), 5 nmol into PC (Figure 3-5 D, pulse) and 1 nmol into CE (Figure 3-5 E, pulse). When E600 was introduced during the pulse labeling it had no effect on the incorporation of fatty acid into PC or TG but significantly decreased the incorporation of the fatty acid into CE (34% decrease, $P<0.04$). (Figures 3-5 and 3-6 were provided by Dr. Richard Lehner).

It is unlikely that E600 inhibited acyl-CoA:cholesterol acyltransferase (ACAT) activity because another esterase inhibitor from the same series of

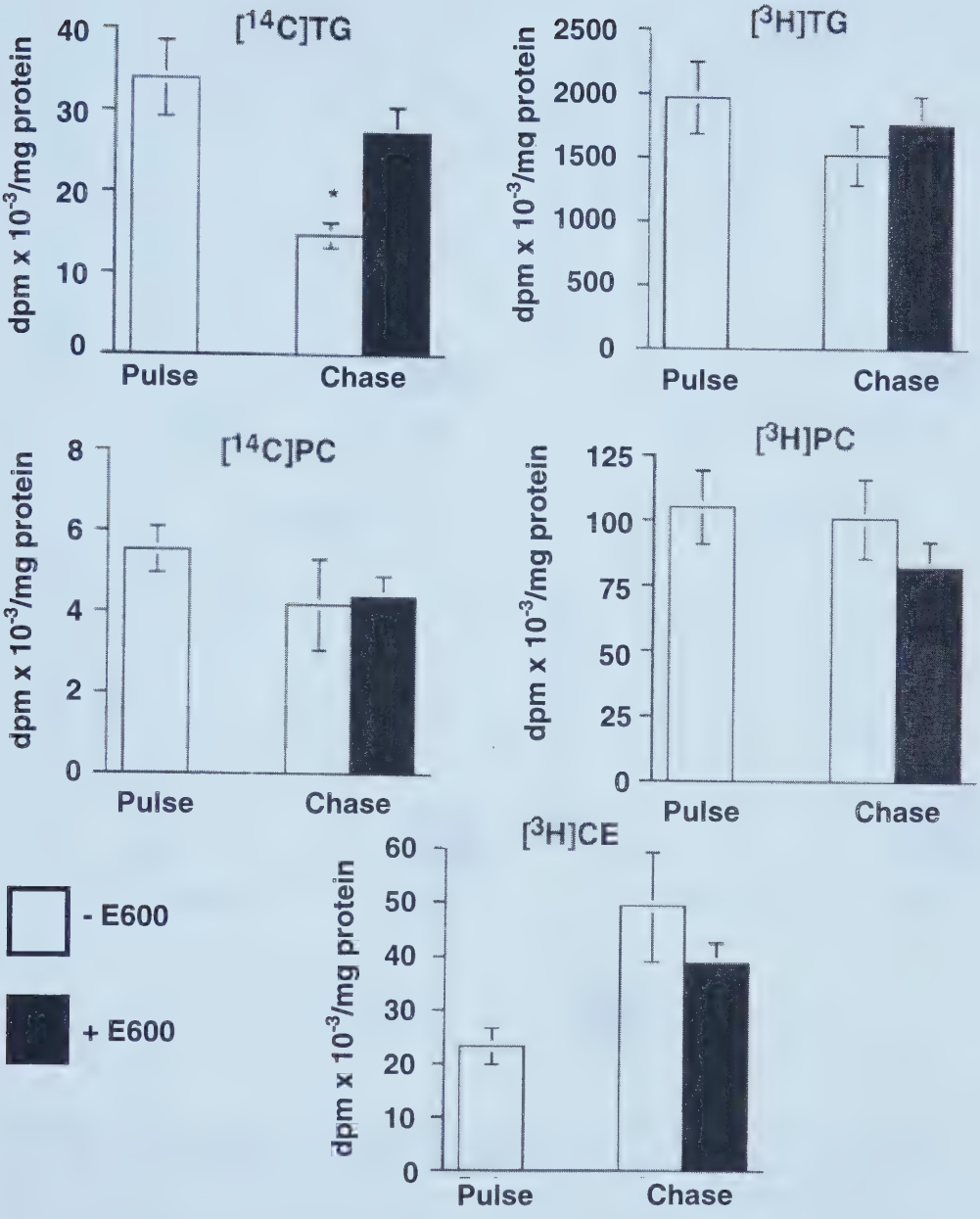


Figure 3-5 Mobilization of lipids in primary rat hepatocytes incubated with or without E600. Hepatocytes were incubated 4 h in the presence of 0.4 mM [^{3}H] oleic acid and [^{14}C] glycerol (pulse). Cells were washed for 1 h in DMEM followed by a second wash ± E600 (100 μM) and subsequently incubated for 4 h ± E600 (100 μM) (chase). Cells were collected, and radioactivity associated with the various lipids was determined as described in Materials and Methods. Each data represents means ± SD from three independent experiments. *P<0.009. TG, triacylglycerol; PC, phosphatidylcholine; CE, cholesteryl ester.

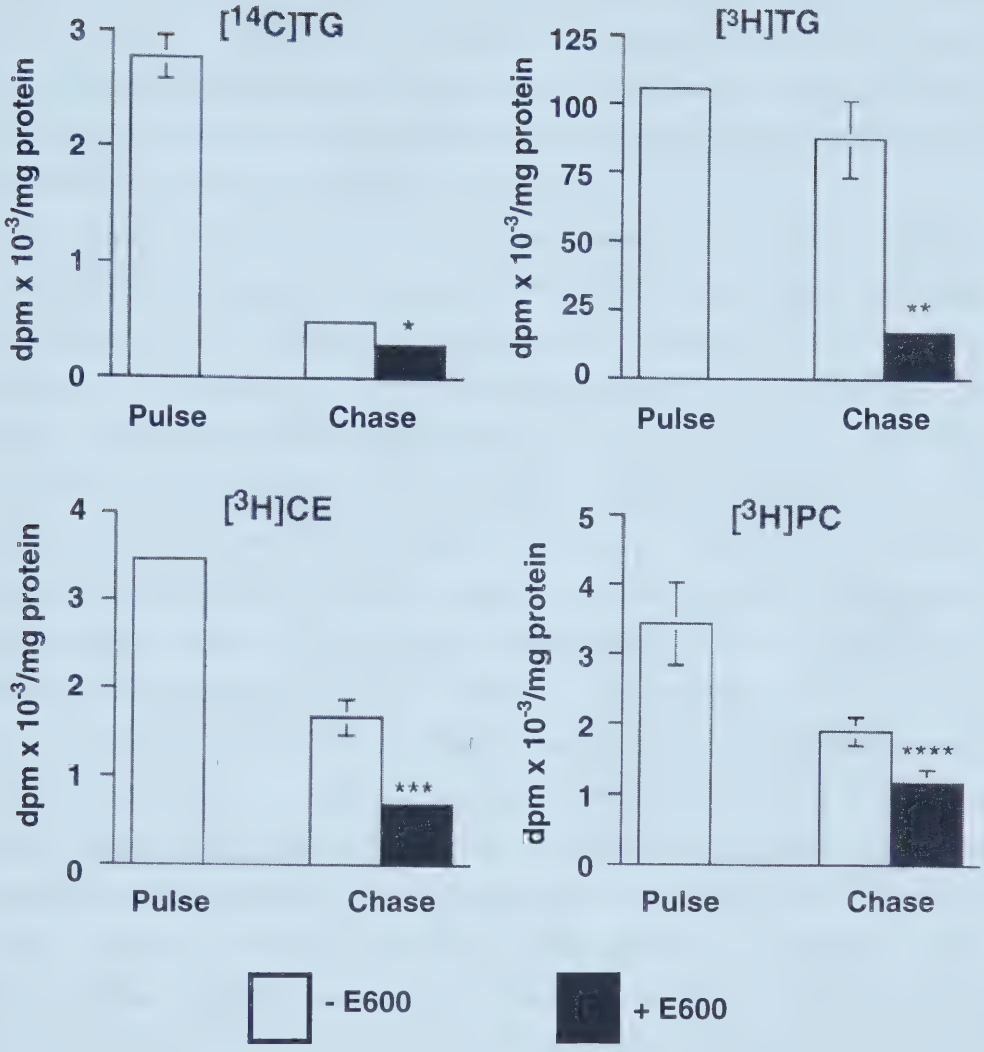


Figure 3-6 Secretion of lipids from primary rat hepatocytes incubated with or without E600. Hepatocytes were incubated 4 h in the presence of 0.4 mM [^{3}H] oleic acid and [^{14}C] glycerol (pulse). Cells were washed for 1 h in DMEM followed by a second wash ± E600 (100 μM) and subsequently incubated for 4 h ± E600 (100 μM) (chase). Media were collected and radioactivity associated with the various lipids was determined as described in Materials and Methods. Data represents means ± SD from three independent experiments. *P<0.015; **P<0.0001; ***P<0.001; ****P<0.005 . TG, triacylglycerol; PC, phosphatidylcholine; CE, cholesteryl ester. This figure was provided by Dr. Richard Lehner.

compounds, bis(4-nitrophenyl)phosphate at concentrations used in these experiments had no effect on ACAT activity *in vitro* (Diczfalusy *et al.*, 1996). Rather, the lower incorporation of fatty acid into CE in the presence of the inhibitor may be related to the inhibition of TG turnover, i.e. the released fatty acid from TG might serve as a substrate for ACAT.

This view is supported by the observation that the levels of radio-labeled intracellular CE more than doubles (from 1 nmol to 2.35 nmol) during the chase period (Figure 3-5 E), when the only source of labeled fatty acid is the pre-labeled pool of lipids (such as TG and phospholipids). Interestingly, the increased cellular levels of labeled CE during the chase period did not lead to increased CE secretion (Figure 3-6 C). Although relatively constant levels (about 5 nmol) of oleate-labeled TG secretion were observed during both pulse and chase periods (Figure 3-6 B), this was not the case for CE or PC. A significantly larger proportion (about 160 pmol) of oleate-labeled CE was secreted in the presence of the extracellular fatty acid compared with the chase period (about 80 pmol), and this was despite the apparent increase of oleate-labeled CE levels during the chase period. The data suggest dissimilar pathways for the assembly of the two neutral lipids into VLDL. Levels of secretion of prelabeled PC were also lower than those obtained in the presence of exogenous oleate (Figure 3-6 D). The relatively constant secretion of oleate-labeled TG (Figure 3-6 B) confirms earlier observations of the preferred utilization of fatty acids incorporated in the TG in storage pools for assembly of VLDL (Wiggins and Gibbons, 1992). Inhibition of the mobilization of preformed TG by E600 dramatically decreased TG secretion (Figure 3-6 B), again demonstrating the important role of TG lipolysis in the assembly and secretion of VLDL. The decrease of TG secretion after treatment of hepatocytes with the lipase inhibitor was confirmed by measurements of TG mass in the media and within the cells. The data presented in Figure 3-7 agree well with those obtained by metabolic radio-labeling. Although about 15% of stored TG was secreted during the 4 h chase in control incubations, less than 5% of stored TG was secreted in the presence of E600.

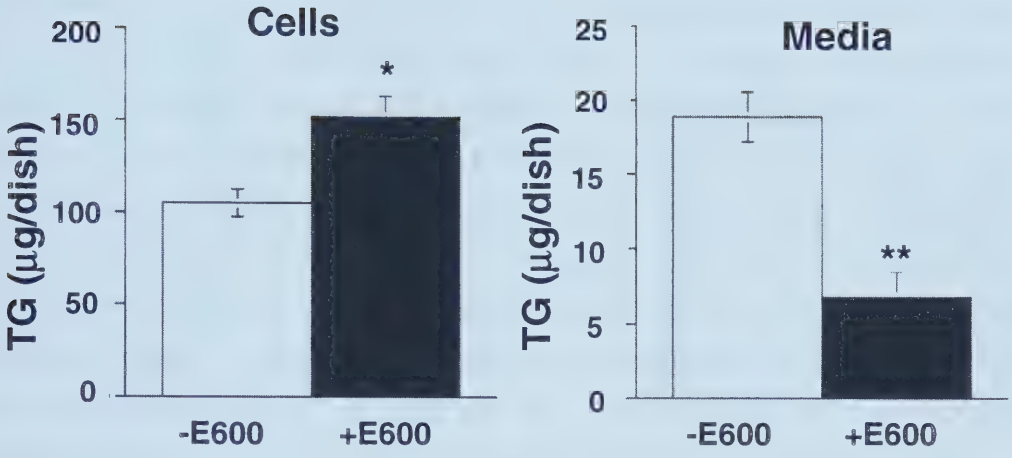


Figure 3-7 Secretion of TG from primary rat hepatocytes incubated with or without E600. Hepatocytes were incubated with 0.4 mM oleate to generate TG stores, washed and incubated 1 h ± E600 (100 µM). Cells were subsequently incubated for 4 h with (black bars) or without (white bars) E600 (100 µM). Media and cells were collected, and TG mass was determined as described in Materials and Methods. Data represent means ± SD from three independent experiments. *$P<0.05$; **$P<0.002$

3.3.5 Inhibition of TGH results in decreased apoB secretion

Because E600 treatment decreased secretion of lipids that are usually components of apoB containing lipoproteins (PC, TG, CE), the effect of E600 on apoB secretion was examined. Rat hepatocytes produce both forms of apoB (apoB100 and apoB48 – see Section 1.1.5). Preincubation of hepatocytes with E600 resulted in decreased secretion of both [^{3}H] leucine-labeled apoB48 (40%, $P<0.02$) and apoB100 (90%, $P<0.0001$) (Figure 3-8 Media). The differences between the observed inhibition of apoB48 versus apoB100 secretion after E600 treatment are not known but may be related to the fact that apoB48 can be secreted as a relatively lipid-poor particle (high density), whereas lipid-poor apoB100 is thought to be secretion-incompetent and undergoes intracellular degradation (Dixon and Ginsberg, 1993; Fisher *et al.*, 1997; Liao *et al.*, 1998; Yao *et al.*, 1997). The data support previous observations from our laboratory where preferential lipidation of apoB100 was observed in McArdle RH7777 cells transfected with TGH cDNA (i.e. increased apoB100 VLDL formation) (Lehner and Vance, 1999).

Analysis of cellular [^{3}H] apoB levels indicated that the inhibitor treatment decreased apoB48 levels by 30% ($P<0.05$) and apoB100 levels by 80% ($P<0.0001$) (Figure 3-8 Cells). Intracellular degradation of apoB in hepatoma cell lines has been postulated to be mediated primarily by the proteasome, however, inclusion of a proteasome inhibitor, lactacystin, during incubations did not significantly increase cellular or secreted apoB levels (Figure 3-8). This suggests that the proteasome may not be the major pathway of apoB degradation in primary rat hepatocytes. The results are in support of recent data showing that in primary hepatocytes, a degradation process within a post-ER compartment rather than in the ER is involved in the loss of apoB100 from Golgi following treatment of the cells with polyunsaturated fatty acids (Fisher *et al.*, 2001).

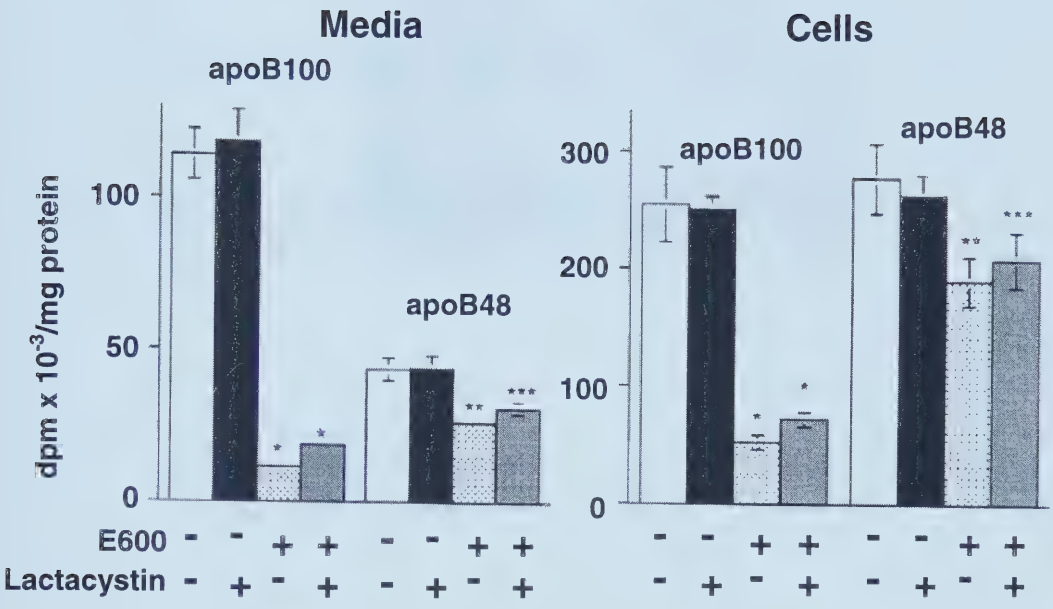


Figure 3-8 The effect of E600 on apoB assembly and secretion from primary rat hepatocytes. Cellular TG levels were raised by incubation of the cells for 4 h with 0.4 mM oleic acid. Cells were then incubated ± E600 (100 μM) for 2 h, followed by 2 h incubation with radio-labeled leucine ± E600 ± lactacystin (10 μM). Cells and media were collected, apoB was immunoprecipitated and the radioactivity associated with the protein was determined following SDS-PAGE, excision of the protein band and scintillation counting as described in Materials and Methods. Each data represents the mean ± SD from three independent experiments.
*$P<0.0001$; **$P<0.02$; ***$P<0.05$
This figure was provided by Dr. Richard Lehner.

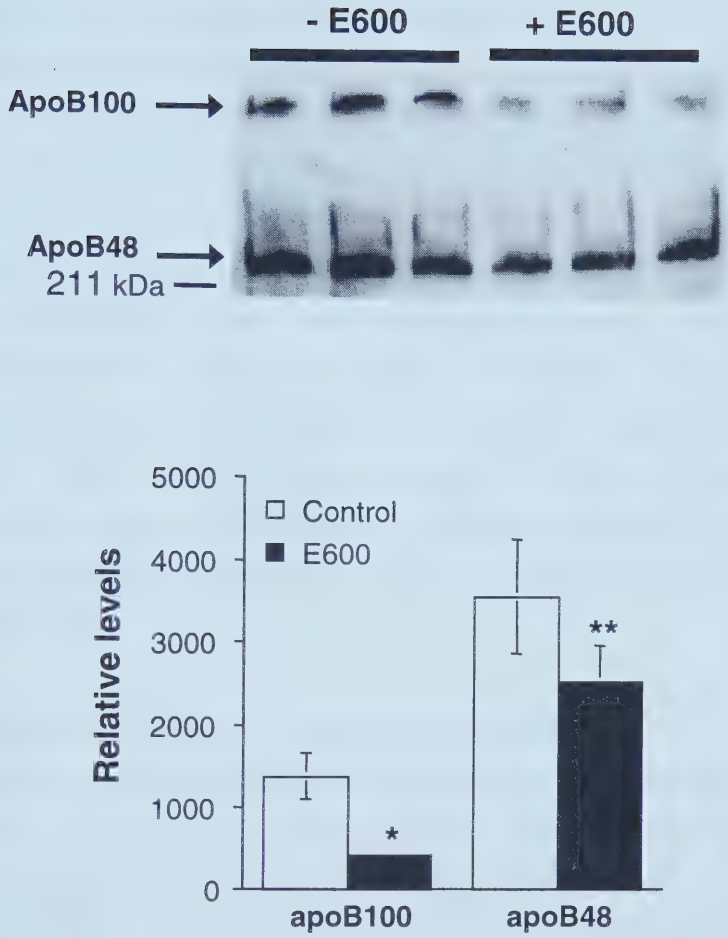


Figure 3-9 The effect of E600 on apoB secretion from primary rat hepatocytes. Hepatocytes were incubated with 0.4 mM oleate to generate TG stores, washed and incubated 1 h ± E600 (100 μM). Cells were subsequently incubated for 4 h ± E600. Media apoB was collected by Cab-O-Sil precipitation and analyzed by immunoblotting as described in Materials and Methods. Top: Immunoblot, Bottom: densitometry of the immunoblot. * $P \leq 0.026$ ** $P \leq 0.11$

Confirmation of the metabolic labeling studies was accomplished by analysis of secreted apoB by immunoblotting. ApoB100 secretion from inhibitor treated cells was substantially decreased (70%) while apoB48 secretion was more resistant to the inhibitor (30% decrease) (Figure 3-9).

It could be argued that E600 may not be a specific TGH inhibitor as it also inhibits other lipases (Moreau *et al.*, 1991). In order to address this issue directly, studies were also performed with a specific TGH inhibitor GR148672X (Figure 3-1). This compound has been shown not to inhibit the activities of pancreatic lipase, hormone sensitive lipase, lipoprotein lipase or acetylcholine esterase (Borg-Capra *et al.*, personal communication). Under the same conditions described for E600 in Figures 3-7 and 3-9, except using 10 μM GR148672X instead of 100 μM E600, 14% of stored TG was secreted in control incubations. This proportion was decreased to 7.5% in the presence of the specific inhibitor (Figure 3-10 Media TG), a decrease of 46% ($P<0.02$). ApoB100 secretion was decreased by 42% ($P<0.03$) and apoB48 by 20% ($P<0.06$) (Figure 3-10 Media apoB).

3.3.6 Inhibition of TGH does not reduce apoB synthesis

To demonstrate that treatment of primary rat hepatocytes with E600 or GR148672X did not reduce synthesis of apoB, metabolic labeling (pulse-chase) experiments were performed with [^{35}S] labeled methionine and cysteine as described by Jiang *et al.* (Jiang *et al.*, 2001). The results in Figure 3-11 demonstrate that neither of these lipase inhibitors affected the synthesis of nascent apoB48 or apoB100. Media apoB levels were nearly undetectable during these pulse-chase experiments, an expected result given the time course of the assembly and secretion of a lipoprotein particle (about 45 min) (Borchardt and Davis, 1987). The inhibitors did not affect the general secretory pathway as assessed by the secretion of albumin (Figure 3-12) and were not detrimental to cell integrity as assessed by the leakage of LDH from the hepatocytes into the media.

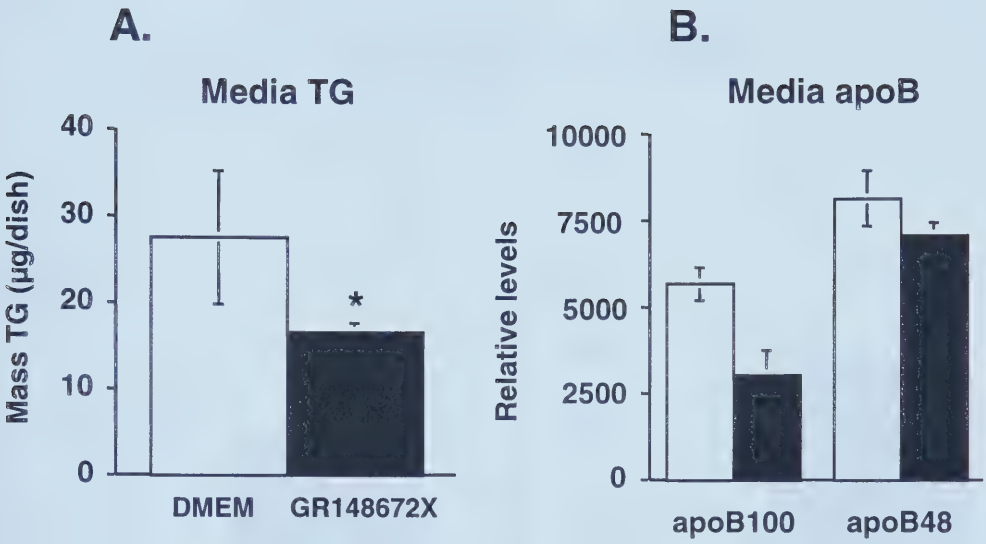


Figure 3-10 The effect of the TGH specific inhibitor GR148672X on secretion of TG and apoB from rat hepatocytes. Hepatocytes were incubated with 0.4 mM oleate to generate TG stores, washed and incubated 1 h ± GR148672X (10 µM). Cells were subsequently incubated for 4 h ± GR148672X (10 µM).

A. Media were collected, and TG contents was determined as described in Materials and Methods. Data represent the mean ± SD from three independent experiments. * $P<0.02$.

B. ApoB in the media was analyzed by Cab-O-Sil precipitation and immunoblot, followed by densitometry as described in Materials and Methods.

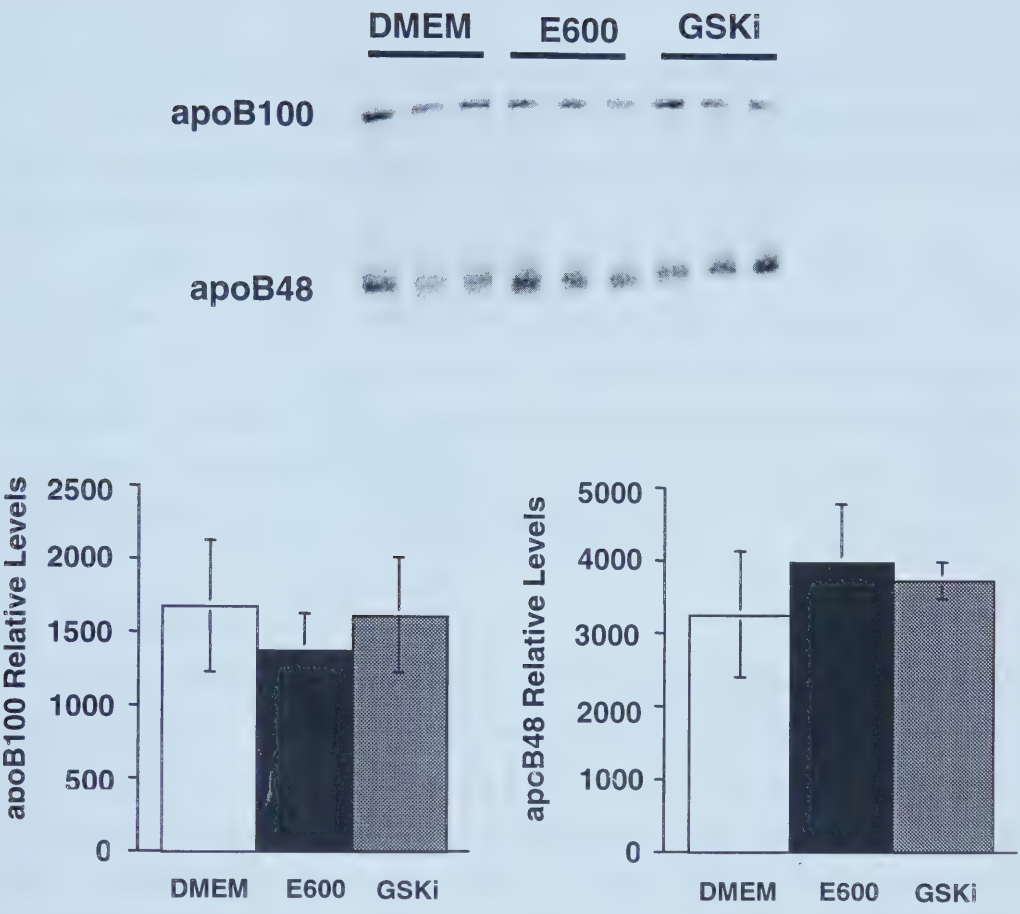


Figure 3-11 Synthesis of apoB by primary hepatocytes treated with lipase inhibitors. Rat hepatocytes were incubated overnight with DMEM containing 15% FBS and 0.4 mM oleic acid / 0.5% BSA. Cells were incubated with ^{35}S-labeled methionine and cysteine with or without E600 (100 μM) or GR148672X (10 μM) (GSKi) as described in Materials and Methods. Cells were harvested in 2 mL of PBS containing protease inhibitors. The apoB was immunoprecipitated from 1 mL aliquots of cell sonicates and media. Samples were resolved via SDS-PAGE and transferred to nitrocellulose membranes. The membranes were exposed to PhosphorImaging screens, and resulting bands were quantitated using Bio-Rad Quantity One software. Densitometry results of the PhosphorImager analysis are shown (means ± SD).

3.3.7 In vivo affects of a TGH specific inhibitor on plasma lipids in hamsters

Acute feeding of hamsters (3 days) with the specific inhibitor GR148672X resulted in significant decreases in plasma TG, VLDL/LDL cholesterol and apoB100 (Figure 3-13 A.).

A single-oral dose of GR148672X (25 mg/kg) or solvent (DMSO) was administrated to hamsters, then plasma lipoproteins were analyzed by HPLC (Figure 3-13 B.). The cholesterol and TG distribution showed that GR148672X decreased the number of VLDL particles without altering the particle composition with respect to TG and CE content (Figure 3-13 C.). The studies in hamsters were performed by collaborators at GlaxoSmithKline.

3.4 Discussion

TG secreted in VLDL particles from the liver is a principal source of energy for muscle or other tissues. The fatty acids that make up VLDL TG are mainly derived from a preformed TG storage pool and only some of this fatty acid is derived from *de novo* synthesis. Recent studies (Aarsland and Wolfe, 1998) have demonstrated that even during induced *de novo* lipogenesis by carbohydrate feeding, the secretion of preformed fatty acids accounted for 99% of total VLDL associated TG secretion before the start of the dietary regimen, and 70% and 79% after one day and 4 days of a lipogeneic diet, respectively. The possible sources of the stored hepatic TG fatty acids are *de novo* synthesized fatty acids, plasma fatty acids derived from adipose tissue TG hydrolysis, TG from remnant particles that are endocytosed or fatty acids from turnover of hepatic membrane lipids. The importance of adipose tissue derived fatty acids for production of hepatic TG stores that would be utilized for VLDL assembly has been recently demonstrated by studies in mice in which the gene coding for hormone-sensitive lipase was ablated (Haemmerle *et al.*, 2002). HSL deficiency resulted in decrease fatty acid delivery to the liver, lower intracellular liver TG concentration and decreased VLDL associated TG secretion.

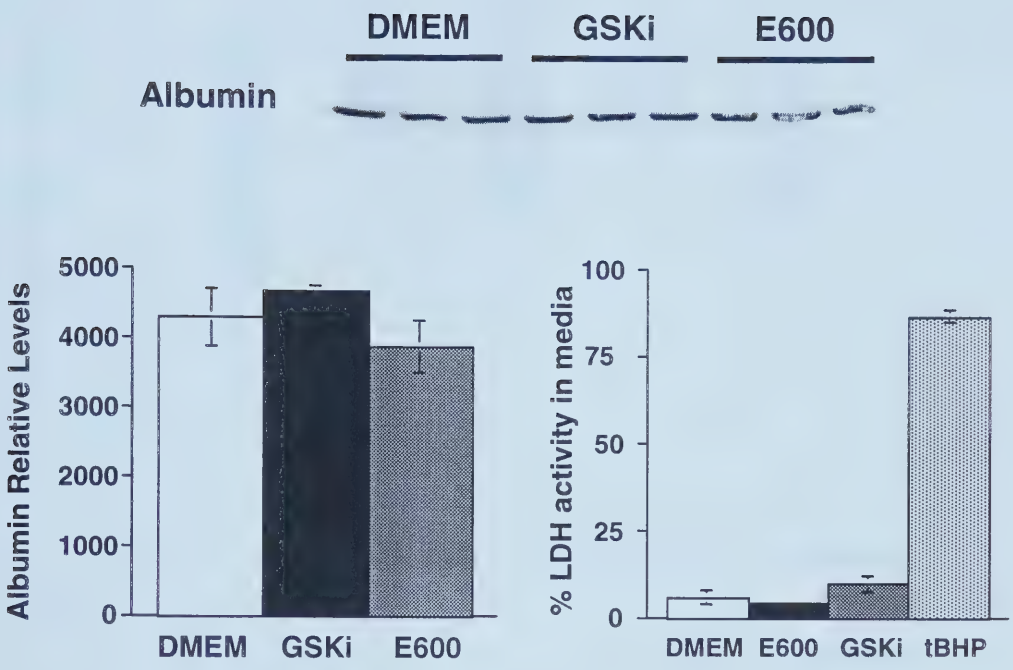
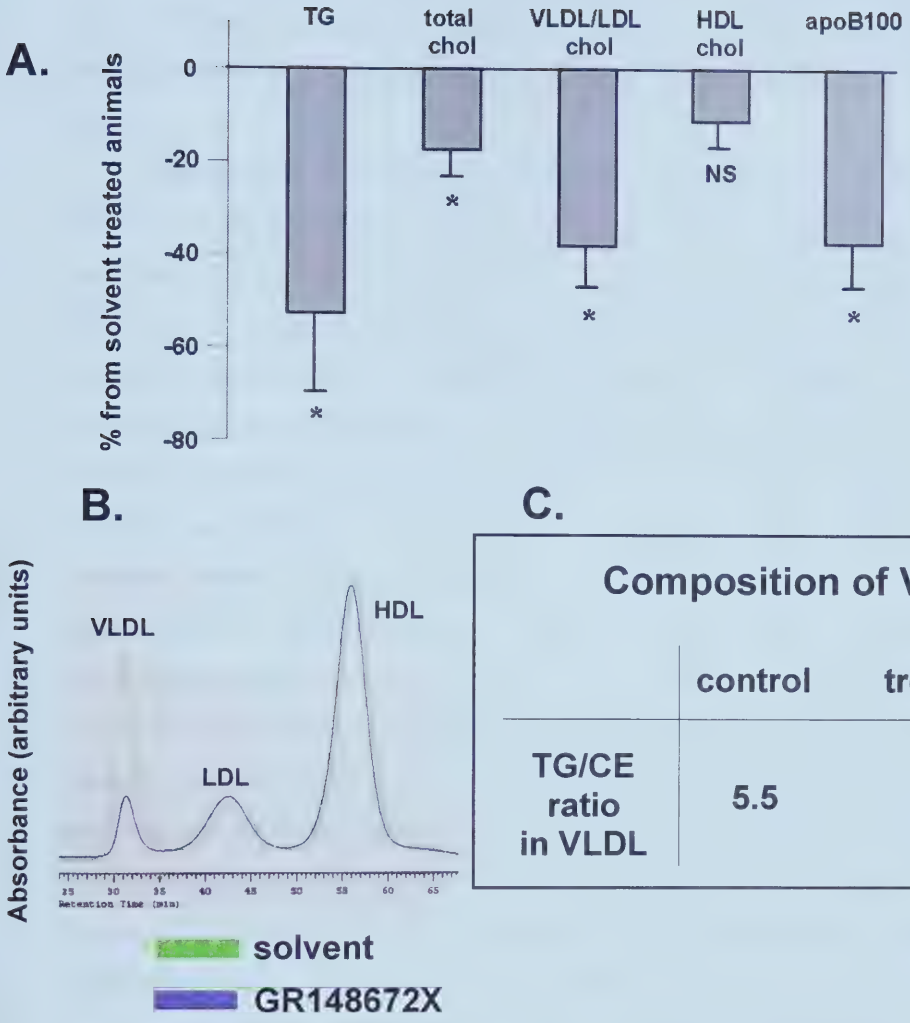


Figure 3-12 Lipase inhibitors do not affect protein secretion or cell integrity. Hepatocytes were treated as in Figure 3-11, except that albumin was immunoprecipitated from 1 mL aliquots of media. PhosphorImager analysis (top) and densitometry of that scan (bottom left) are shown (means ± SD).

Cell Integrity. Hepatocytes were incubated with 0.4 mM oleate to generate TG stores, washed, and incubated 1 h ± 100 μM E600 or 10 μM GR148672X (GSKi). Cells were subsequently incubated for 4 h ± 100 μM E600 or 10 μM GSKi. Some cells incubated without lipase inhibitors were incubated with DMEM for 3 h followed by DMEM containing 400 μM *tert*-butyl hydroperoxide (tBHP) for 1 h. Aliquots of medium were collected, and cells were scraped into 1 ml of PBS containing protease inhibitors. Lactate dehydrogenase (LDH) activity was determined as described in Materials and Methods. Data represent means ± SD from three dishes of cells.



Composition of VLDL

	control	treated
TG/CE ratio in VLDL	5.5	5.3

Figure 3-13 Effect of GR148672X on plasma lipids and apoB100.
Hamsters fed *ad libitum* were given GR148672X or solvent alone orally for 3 days (**A.**) or in a single administration (**B.** and **C.**) as described in Materials and Methods. Four hours after the last administration of the compound or solvent, animals were sacrificed and plasma lipids and apoB levels analyzed.
A. Summary of plasma lipids and apoB100 measured in animals receiving the TGH inhibitor compared to animals receiving solvent alone.
B.: example of an HPLC trace of plasma cholesterol.
C.: Ratio of triacylglycerol (TG) and cholesteryl ester (CE) found on VLDL particles from hamsters treated ± GR148672X.
Figure provided by Borg-Capra *et al.*, GlaxoSmithKline.

Hepatic TGH protein levels in HSL-null mice are not changed, which suggests that mobilization of hepatic TG stores in these mice may not be affected, and the decreased VLDL production was correlated to the amount of intrahepatic TG stores.

Microsomal membranes harbour the majority of neutral TG lipase activity in liver (Lehner and Verger, 1997). Only about 10% of hepatic TG lipase activity was detected in the soluble cytosolic fraction. On purification of the lipolytic activity from hepatic microsomes, most of the activity co-purified with TGH. The results shown in this Chapter indicated that a specific inhibitor of TGH decreases VLDL (TG and apoB) secretion. Based on this evidence, a model can be proposed in which the substrate pool for TGH in the liver exists in lipid droplets found in the ER lumen (Figure 1-12). Theses droplets may be derived from cytosolic stores via a process that involves microsomal triglyceride transfer protein (MTP). MTP has been shown to be required for secretion of TG-rich apoB containing lipoproteins. MTP is necessary for proper folding of apoB (reviewed in (Fisher and Ginsberg, 2002; Shelness and Sellers, 2001)). From recent studies in mice in which the MTP gene was disrupted (Raabe *et al.*, 1999), and through MTP inhibition studies (Kulinski *et al.*, 2002; Wang *et al.*, 1999b), it became apparent that MTP function may be required for the formation of a lumenal TG storage droplet (see Section 1.3.3). The lumenal droplet may be the primary source of VLDL TG. It has been hypothesized that the lumenal droplet may "fuse" with the nascent, TG-poor primordial apoB particle to form fully lipidated VLDL, however, there are no data that would directly support the occurrence of such a fusion. In the proposed model, lipolysis of the lumenal droplet by TGH to partial acylglycerols and fatty acids would provide substrates for TG resynthesis by lumenally localized acyltransferases followed by TG loading onto nascent apoB containing particles. Alternately, TGH may access TG at the inner ER membrane as TGH has been shown to adsorb to lipid monolayers and contains a "lipid binding domain" (Figure 1-9) that may promote interactions with hydrophobic surfaces such as membranes or the surface of lipid droplets. Upon contact with the ER, TGs could egress from the cytosolic lipid

droplet into the ER bilayer. Bilayers can incorporate up to 5 mol% TG without affecting the membrane integrity. However, it is difficult to conceptualize that TG already within the bilayer would need to be hydrolyzed and re-esterified in order to be assembled into an apoB containing particle.

Another putative function of TGH would be the delipidation of secretion-incompetent or misfolded apoB particles, facilitating their degradation. The idea behind this hypothesis is that misfolded apoB particles could potentially interfere with the production of correctly assembled lipoproteins by sequestering chaperones including MTP. However, TGH inhibitor treatment did not result in increased cellular apoB levels. In addition, the data presented in Chapter 5 demonstrates that apoB associated TG is not a substrate for TGH. It is important to note that inhibition of TGH did not affect the secretion of albumin (Figure 3-12), indicating our results were not due to a general effect of the compounds on protein translation or on the secretory pathway.

Several reversible inhibitors of TGH were identified by a chemical proteomic strategy known as activity based protein profiling. These assays found that alkyl/aryl trifluoromethyl ketones were both selective and potent chemical inhibitors of TGH (Leung *et al.*, 2003). Others identified tacrine derivatives that selectively inhibit human TGH but not other carboxylesterases (Bencharit *et al.*, 2003a). The therapeutic potential of these carboxylesterase inhibitors are unknown.

3.4.1 Conclusions

Inhibition of microsomal lipolytic activity in intact hepatic cells resulted in dramatic decreases of apoB and TG secretion. The data further support the concept that the lipolysis/re-esterification pathway represents a regulated step in VLDL assembly. In addition, the inhibitors substantially reduced CE and PC secretion. Analysis of secreted apolipoproteins indicated that apoB100 secretion was much more sensitive to lipase inhibition than apoB48, perhaps owing to the ability of apoB48 to be secreted as a relatively lipid-poor particle. The results agreed with those obtained in hepatoma cells transfected with the TGH cDNA

where preferential lipidation of apoB100 was observed. TGH may therefore be an attractive target for pharmacological intervention that would decrease the levels of atherogenic lipoproteins in plasma.

Chapter 4:

Subcellular localization of TGH

The majority of the information presented in this Chapter has been published in Gilham, D., M. Alam, W. Gao, D.E. Vance, and R. Lehner. (2005) Triacylglycerol hydrolase is localized to the endoplasmic reticulum by an unusual retrieval sequence where it participates in VLDL assembly without utilizing VLDL lipids as substrates. *Mol. Biol. Cell.* 16:984-996, and is adapted into this Thesis with permission

4.1 Introduction

Soluble proteins that are resident in the lumen of the ER are retrieved from the traffic of secreted proteins from an early Golgi compartment (reviewed in (Pelham, 1991)). In animal cells, this process is mediated by a C-terminal consensus sequence KDEL (Munro and Pelham, 1987). Several liver microsomal carboxylesterases from human, rat and rabbit carry variants of the KDEL consensus ER retention sequence at their extreme C-termini of the type HXEL that have been shown to confer ER retrieval (Robbi and Beaufay, 1991). Higher eukaryotes appear to tolerate considerable variation at the "X" residue. Why these proteins (and specifically this family of proteins) have diverged from carrying the consensus KDEL sequence for ER retention is presently unclear. The human TGH enzyme bears HIEL at its extreme C-terminus. Expression of a secreted and functional carboxylesterase has been accomplished by mutation of similar C-terminal sequences (Oosterhoff *et al.*, 2002; Robbi and Beaufay, 1991; Scott *et al.*, 1999). To determine if the HIEL motif is the only determinant of TGH ER retention in mammalian cells, a deletion mutant was produced via PCR that lacked the coding region for these amino acids (ΔR-TGH). The ΔR-TGH protein was produced in McArdle RH7777 cells, and appearance of the protein and lipase activity in the media was monitored.

The C-terminal sequence of TGH may be important in directing this protein to a region within the ER. Immunogold electron microscopy in transfected cells revealed a disparate distribution of this enzyme within this organelle, with particularly intense localization in regions surrounding mitochondria (Lehner *et al.*, 1999). TGH distribution within the ER may be critical for its efficient participation in VLDL assembly as it could dictate proximity to substrate pools or apoB. Juxtaposition to these entities could impact the ability of TGH to direct lipids towards assembly of nascent VLDL. Investigation was performed to determine whether the localization of TGH within the ER is influenced by a C-terminal sequence thought to retain TGH within this compartment, and if secretion of neutral lipid in transfected McArdle RH7777 cells is affected by mutation of this sequence.

4.1.1 Cloning of the full length human triacylglycerol hydrolase cDNA

The full-length human TGH cDNA had been cloned from a human liver cDNA λgt11 phage library by our laboratory prior to initiation of this study (Alam *et al.*, 2002a). In brief, isolation of this cDNA began with the design of two 40-mer oligonucleotide primers from highly conserved regions between mouse, rat, and pig TGH. The sequences of these primers were:

Forward: 5'-GCA TCT GGG GAT TCT TCA GCA CAG GGG ATG AAC ACA GCC G-3'

Reverse: 5'-GAG CAA AGT TGG CCC AGT ATT TCA TCA CCA TTT TGC TGA G-3'

The primers were successfully used to amplify a 1-kb fragment from the cDNA library by PCR. The product was sequenced, and this information used to probe existing databases. The sequence was identical to human carboxylesterase I (CESI; GenBank accession number NM_001266). Further, this identification was consistent with N-terminal sequencing that determined the first 20 amino acid residues from TGH purified from human liver. Purification from human liver was performed by collaborators at GlaxoSmithKline using the procedure described by Lehner and Verger (Lehner and Verger, 1997). Their results from N-terminal sequencing were conveyed to our laboratory via a personal communication. Using this information, primer sequences were designed according to the 5' and 3' regions of hCESI. Subsequently the full length human TGH cDNA (of approximately 1.7 kb) was amplified by PCR. The primer sequences were:

Forward: 5'-AAC TGT CGC CCT TCA CGA TGT G-3'

Reverse: 5'-TCA CAG CTC TAT GTG TTC TGT CTG G-3'

The PCR product was ligated to pCR2.1TOPO (Invitrogen) to obtain cloning sites and sequenced to ensure fidelity. The insert was then subcloned into the mammalian expression plasmid pCI (Promega). The human TGH cDNA was stably expressed in liver derived McArdle RH7777 cells by calcium phosphate-mediated transfection, and individual colonies derived by selection

with Geneticin. Expression of human TGH protein in the various cell lines obtained was confirmed by immunoblotting and activity assays.

4.1.2 Functional motifs predicted in the human TGH primary amino acid sequence

The open reading frame of the human TGH cDNA encodes a protein of 568 amino acids or approximately 60 kDa (Gilham and Lehner, 2004). As indicated in Figure 4-1, several motifs and consensus sequences in the TGH protein are predicted upon entering the human TGH primary amino acid sequence into database queries (e.g. http://www.embl-heidelberg.de/predictprotein/predictprotein.html). Several of these predicted elements became the focus of investigation in our laboratory. TGH has an amino-terminal 18 amino acid signal peptide that would presumably direct the protein to the rough ER for synthesis and get cleaved by signal peptidases present in the lumen. Cleavage of the signal sequence was demonstrated by amino-terminal sequencing of both the purified rat and human TGH proteins ((Lehner and Vance, 1999) and personal communication from GlaxoSmithKline). As indicated in Figure 1-7, the residues predicted to from the catalytic triad are highly conserved between orthologous TGH proteins. Participation of Ser 221, Glu 354 and His 468 in the TGH catalytic triad was demonstrated by site directed mutagenesis and expression in Sf-9 insect cells. Mutation of any of these residues resulted in catalytically inactive proteins (Alam *et al.*, 2002b). A potential *N*-glycosylation site was identified at Asn 79. The requirement for glycosylation is investigated in Chapter 6. There are also several consensus sequences for phosphorylation sites, which are investigated in Chapter 6. The sequence also contains five Cys residues. Four of these are predicted to participate in two disulfide bridges, leaving a free cysteine to engage in intermolecular covalent interactions or acylation. These possibilities are addressed in Chapter 6. A lid domain is thought to exist in residues 87-116 and regulate substrate specificity and access to the active site.

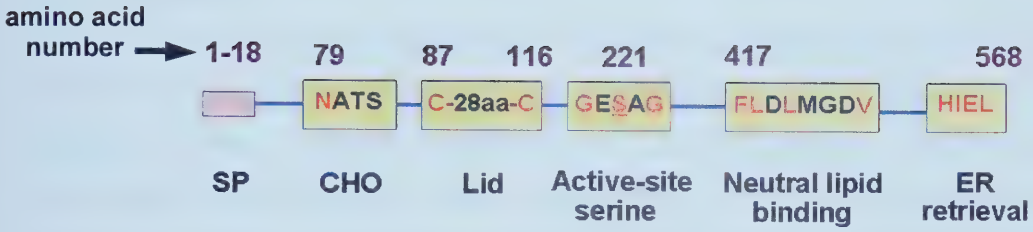


Figure 4-1 Motifs predicted in the human TGH amino acid sequence.
Functional motifs were predicted via database searches of human TGH.
SP: signal peptide, CHO: *N*-glycosylation

Finally, a hydrophobic stretch of amino acids is found between residues 417-424 that may be involved in lipid binding. A C-terminal HIEL sequence is present and potentially responsible for the retention of TGH within the ER lumen (Robbi and Beaufay, 1991). The functionality of this sequence is investigated here in the current Chapter.

4.2 Materials and Methods

4.2.1 Reagents

TRIZOL Reagent, Superscript II reverse transcriptase and the plasmid pBudCE4.1 and were purchased from Invitrogen Canada, Burlington, ON, Canada. Prolong Antifade and fluorescently labeled secondary antibodies were purchased from Molecular Probes, Eugene, OR. IRDye 800 conjugated anti-rabbit IgG secondary antibodies were from Rockland Immunochemicals, Gilbertsville, PA. Monoclonal antibody specific for the flag epitope was purchaced from Stratagene, La Jolla, CA. Polyclonal antibodies for TATA binding protein were from Santa Cruz Biotechnology, Santa Cruz, CA. Monoclonal antibody for the myc epitope was obtained from Clontech, Palo Alto, CA, while polyclonal antibodies for calnexin and monoclonal antibody for BiP (also known as Grp78) were obtained from Stressgen Biotechnologies, Victoria, BC, Canada. Paraformaldehyde, as well as rabbit polyclonal antibodies for the myc epitope and 10 nm-gold conjugated anti-rabbit IgG were from Sigma-Aldrich, Oakville, ON, Canada. All other chemicals and reagents were acquired from local suppliers and were of the highest quality available.

4.2.2 Production of ΔR-TGH

A deletion mutant of TGH that lacks the coding region for the C-terminal four amino acids (-HIEL) was generated by PCR using the forward primer: 5'-TAC TGT CAC GCT CTC GAG ATG TGG CTC CGT GCC TTT ATC-3' and the reverse primer: 5'-TGA CGT TAG CTT GGG TAC CTC ATT CTG TCT GGG GTG GCT TCT C-3' and the full length TGH cDNA (Alam *et al.*, 2002a) as a template. The PCR product was cloned into the pCI-neo mammalian expression

vector and termed ΔR-TGH. The PCR and cloning of ΔR-TGH were performed by Dr. Mustafa Alam.

4.2.3 Reverse transcriptase PCR of ΔR-TGH

RNA from 60 mm diameter dishes of 80% confluent ΔR-TGH expressing McArdle RH7777 cells was isolated using TRIZOL Reagent according to the manufacturer's instructions. Total RNA was reverse transcribed using oligo dT_{18} primer and Superscript II reverse transcriptase. A 1 kilobase pair region of ΔR-TGH was amplified using the following primers: forward: 5'-GCA TCT GGG GAT TCT TCA GCA GGG ATG AAC ACA GCC G-3' reverse: 5'-GAG CAA AGT TGG CCC AGT ATT TCA TCA CCA TTT TGC TGA G-3'. Selected amplicons were purified and sequenced to verify their identity as *bona fide* TGH amplicons. Cyclophilin was amplified as previously described (Agellon *et al.*, 2002) and employed as a control for the RT-PCR protocol. Primers used to amplify cyclophilin had the sequence: forward: 5'-TCC AAA GAC AGC AGA AAA CTT TCG-3' reverse: 5'-TCT TCT TGC TGG TCT TGC CAT TCC-3'.

4.2.4 Characterization of ΔR-TGH

In 60 mm diameter dishes, $1.5x10^6$ cells each of untransfected McArdle RH7777 cells, and McArdle RH7777 cells stably transfected with *wt*-TGH, ΔR-TGH or empty pCI-neo were allowed to settle and attach to the dishes overnight. Cells were then incubated 6 h in 2 mL of serum free DMEM. The media were collected and centrifuged at 600 x*g* for 2 min to remove cell debris. Cells were harvested into buffer containing 50 mM Tris pH 7.4, 1 mM EDTA and 250 mM sucrose. The cell suspensions were briefly sonicated and microsomes prepared from post mitochondrial supernatants (Lehner and Kuksis, 1992). Microsomes were resuspended in 1 mL of PBS containing Complete® protease inhibitors (Roche Diagnostics). Seventy μL of media and 35 μL of resuspended microsomes were electrophoresed in denaturing SDS-PAGE (10%), then transferred to a nitrocellulose membrane. TGH and albumin were probed by immunoblotting with anti-human TGH (Alam *et al.*, 2002a) and anti-rat albumin

polyclonal antibodies generated in our laboratory and the laboratory of Dr. Dennis Vance, respectively.

4.2.5 Specific enzyme activity of TGH and TGH mutants

ΔR-TGH was demonstrated to retain lipolytic activity after secretion from cells, however, the four amino acid deletion may have reduced specific enzyme activity. The specific enzyme activity of ΔR-TGH compared to *wild-type* TGH is demonstrated in Chapter 6 (Figure 6-5) along with other TGH mutants introduced in this investigation.

4.2.6 Mutation of the TGH C-terminus to KDEL

A TGH mutant bearing the amino acid sequence -KDEL at its C-terminus, rather than -HIEL, was generated exactly as ΔR-TGH, except using the reverse primer: 5'-TGA CGT TAG CTT GGG TAC CTC ACA GCT CAT CCT TTT CTG TCT GGG GTG GCT T-3'. After ligation into the pCI-neo plasmid, the entire insert was sequenced to ensure integrity, then used to stably transfect McArdle RH7777 cells as per Section 2.6. The PCR and cloning of KDEL-TGH were performed by Dr. Mustafa Alam.

4.2.7 Access of KDEL-TGH versus wt-TGH to endogenous substrate

$1.6x10^6$ untransfected McArdle RH7777 cells or cells stably transfected with either *wt*-TGH or KDEL-TGH were plated on 60 mm diameter dishes and grown overnight. These cells were subsequently incubated in DMEM containing 0.4 mM oleate complexed to 0.5% essentially fatty acid free BSA (see Section 2.7) for 2.5 h to promote TG deposition. [^{3}H] Oleate at 5 μCi/dish was included as a tracer. Cells were washed 3x 10 min with DMEM containing 0.5% BSA to remove labeled fatty acids that were non-specifically associated with the exterior of the cells. Cells were then incubated 4 h in 2 mL of DMEM. Media were collected and cleared of cell debris by centrifugation at 1,700 x*g* for 2 min. Cells were harvested in 2 mL of PBS and sonicated. Lipids from 1 mL aliquots of cells and media were extracted in 4 mL chloroform/methanol (2:1) (Folch *et al.*, 1957)

containing non-radiolabeled lipid carriers as per Section 2.8. Extracted lipids were resolved by TLC and the associated radioactivities measured by scintillation counting as per Section 2.8. Protein concentration in the cell sonicates was determined, and a lipase assay was performed with 4 μg of protein from each sample using the fluorescent based assay described in Section 2.9. Sixty μg of protein were used in SDS-PAGE (10%) and immunoblots were performed using anti-TGH antibodies as above, and anti-TATA binding protein antibodies according to manufacturer's instructions.

4.2.8 Electron microscopy of TGH in McArdle RH7777 cells

The subcellular localization of TGH was assessed by immunogold electron microscopy. The ultrathin frozen sections of *wild type* and TGH-transfected cells were obtained and processed essentially as described by Cui *et al.* (Cui *et al.*, 1993). Ultrathin cryosections were incubated with affinity-purified anti-TGH polyclonal antibodies. Primary antibodies were revealed by incubation for 1 h with colloidal gold 10 nm-gold conjugated anti-rabbit IgG. Sections were subsequently stained with 2% uranyl acetate for 30 min and lead citrate for 5 min and examined in a Hitachi H-7000 electron microscope.

4.2.9 Production of myc-tagged KDEL-TGH and flag-tagged wt-TGH

Sequences for myc and flag epitope tags were introduced to the *wt*-TGH cDNA by PCR using the common forward primer:

5'- cg **gaa ttc** ATG tgg ctc cgt gcc ttt atc c-3'.

Reverse primers were

5'-g ctg gat ctt cat **tct aga** TCA cag ctc tat gtg CTT ATC GTC GTC ATC CTT GTA ATC ttc tgt ctg ggg tgg ctt ctc cac tgc-3' to introduce the flag sequence immediately upstream of the region coding for -HIEL and

5'-g ctg gat ctt cat **tct aga** TCA cag ctc tat gtg CAG GTC CTC CTC TGA GAT CAG CTT CTG CTC ttc tgt ctg ggg tgg ctt ctc cac tgg-3' to incorporate the myc-tag. Shown in bold are restriction enzyme sites engineered into the primers to facilitate subsequent cloning procedures. Upper case and underlined indicate

start (forward primer) and stop codons (reverse primers). Upper case (no underline) indicates the region of the primer coding for the epitope tags. The resulting PCR products were ligated into pCI-neo. The region coding for the C-terminus of the myc-tagged construct was then mutated to myc-KDEL via site directed mutagenesis. The primers used were
forward: 5'-g gag gac ctg AAA GAC gag ctg tga aga tct gtc gac ccg gg-3' and
reverse: 5'-cc cgg gtc gac aga tct tca cag ctc GTC TTT cag gtc ctc c-3'. Mutated codons in the primers are shown in upper case. The cDNAs were sequenced to ensure unwanted errors were not introduced during PCR or mutagenesis. The pBudCE4.1 vector has two promoters and two cloning sites. The myc-tagged KDEL-TGH and flag-tagged *wt*-TGH constructs were excised from pCI-neo using restriction endonucleases, purified from an agarose gel and ligated into pBudCE4.1. This plasmid containing both TGH constructs, as well as empty pBudCE4.1 for control experiments, were used to stably transfect McArdle RH7777 cells as per the Lipofectamine2000 protocol given in Section 2.6.

4.2.10 Colocalization of wt-TGH and KDEL-TGH with each other and with neutral lipid droplets

Transfected McArdle RH7777 cells were grown on sterile cover slips overnight in 6 well plates ($2x10^5$ cells/well). Cells were cultured in serum free medium for 1 h to facilitate removal of immunoglobulins before staining, then fixed to cover slips with 4% paraformaldehyde. Cells were permeabilized with 0.2% Triton X-100 for 5 min and incubated for 1 h with the primary antibodies indicated in the Figure Legends diluted 1:100-1:1,000 with 3% BSA in PBS. After washing, cells were incubated with fluorescently labeled secondary antibodies (either fluorescein, Alexa488 or Texas Red) directed at the appropriate IgG species for 1 h. Cover slips were mounted on microscope slides with Prolong Antifade. When observing colocalization with neutral lipid droplets, cells were incubated with 0.4 mM oleate complexed to 0.5% fatty acid free BSA for 4 h before fixing them to cover slips. Neutral lipid droplets were stained with Nile Red diluted 1:1,000 for 10 min after antibody staining had been completed.

Images were produced using a Zeiss LSM510 confocal microscope with an argon laser delivering a wavelength of 488 nm to excite fluorescein or Alexa488 and a HeNe laser delivering 543 nm to excite Texas Red or Nile Red.

4.2.11 Specific enzyme activity of wt- and KDEL-TGH

In order to determine if the –KDEL mutation engineered in TGH affected enzymatic activity, *in vitro* lipase assays were performed on cell lysates with normalized amounts of TGH. McArdle RH7777 cells stably expressing *wt*-TGH, KDEL-TGH or stably transfected with empty plasmid were grown to approximately 80% confluence on 60 mm dishes. Cells were scraped into PBS, lysed by brief sonication and protein concentration determined. SDS-PAGE was used to separate 45 μg of protein from each cell lysate. Proteins were transferred to a nitrocellulose membrane. Immunoblots were performed using anti-TGH polyclonal antibodies as above and IRDye 800 conjugated anti-rabbit IgG secondary antibodies according to the manufacturer's instructions. Fluorescence intensity for the TGH band in each lane was quantitated using a Li-Cor Odyssey Infrared Imaging System. Cell lysate containing equal amounts of TGH (*wt*-TGH or KDEL-TGH) were used in lipase activity assays.

4.3 Results

4.3.1 A carboxy-terminal tetrapeptide is responsible for microsomal retention of TGH

In order to assess the mechanism of ER retention for TGH, a deletion mutant was constructed that lacks the coding region for the C-terminal four amino acids "HIEL" (ΔR-TGH). The construct was expressed in McArdle RH7777 cells, a cell line that does not endogenously express TGH (Lehner and Vance, 1999).

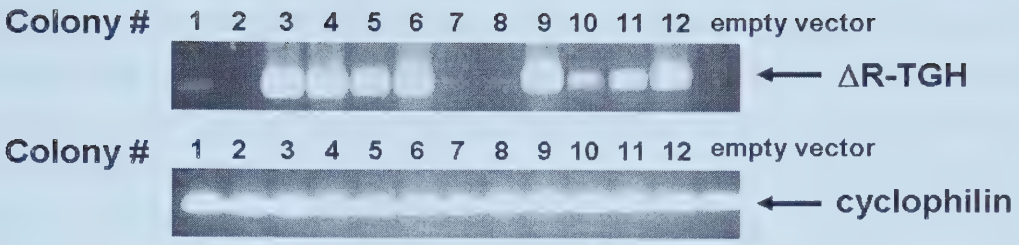


Figure 4-2 Transfection of McArdle RH7777 cells with ΔR-TGH. The cDNA for human TGH lacking the coding region for the carboxy-terminal four amino acids (ΔR-TGH) was stably produced in McArdle RH7777 cells. Reverse transcriptase-PCR was used to detect endogenous cyclophilin or transfected TGH mRNA from resulting cell lines (30 cycles).

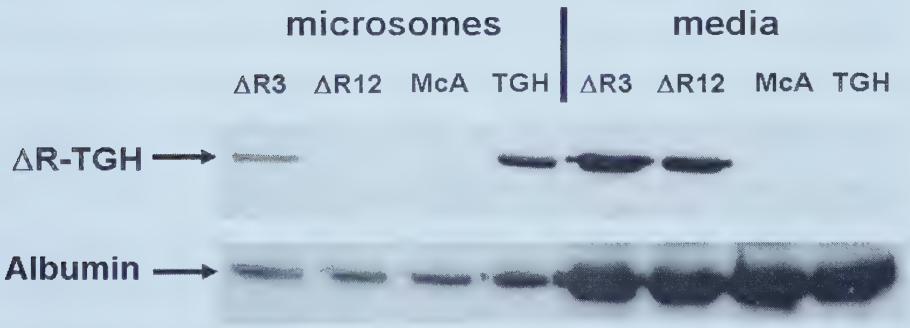


Figure 4-3 A carboxy-terminal sequence is responsible for retention of TGH. Immunoblots using anti-TGH and anti-albumin polyclonal antibodies to probe microsomes or media from McArdle RH7777 cells stably expressing ΔR-TGH, *wt*-TGH or untransfected cells (McA) after an overnight incubation.

After stable transfection, a RT-PCR product could be detected in several of the resulting cell lines using TGH specific primers (Figure 4-2). Clones 3 (ΔR3-TGH) and 12 (ΔR12-TGH) were analyzed further. An immunoreactive species was detected in the media and in isolated microsomes from cell lines developed from both of these colonies (Figure 4-3). Cells transfected with *wt*-TGH contained TGH in microsomes, but did not secrete the protein into the media, indicating that the C-terminal 4 amino acid sequence was responsible for microsomal retention. A dramatic increase in lipase activity was also seen in the media from ΔR3-TGH and ΔR12-TGH expressing cells compared to cells transfected with *wt*-TGH, the empty plasmid or to untransfected cells (Figure 4-4). Collectively these data demonstrate that ΔR-TGH can be secreted as a functional enzyme, and also indicate that the microsomal environment is not required for enzymatic activity.

4.3.2 KDEL-TGH is less effective in mobilizing neutral lipids for VLDL secretion than wt-TGH

TGH co-fractionates with ER elements and lipid droplets (Lehner *et al.*, 1999). Its location was further defined by immunogold electron microscopy. The electron micrograph in Figure 4-5 was produced by analysis of McArdle RH7777 cells stably transfected with *wt*-TGH. It illustrates that TGH is not evenly distributed throughout the ER, but rather is found in cisternae in proximity to mitochondria. Because of this non-uniform distribution of TGH in the ER, the speculation arose that TGH may be targeted to a subdomain of this organelle that is specialized in VLDL production or has better ability to channel lipids towards VLDL assembly and secretion.

In order to investigate whether the C-terminal HIEL sequence allows better access to lipid substrates, a mutant of TGH was created that bears the consensus for ER retention in animal cells at the extreme C-terminus, i.e. KDEL, and McArdle RH7777 cells lines stably expressing this mutant were generated. The immunoblot in Figure 4-6 A indicates that the KDEL-TGH transfected cells used in these experiments express more TGH protein compared to cells

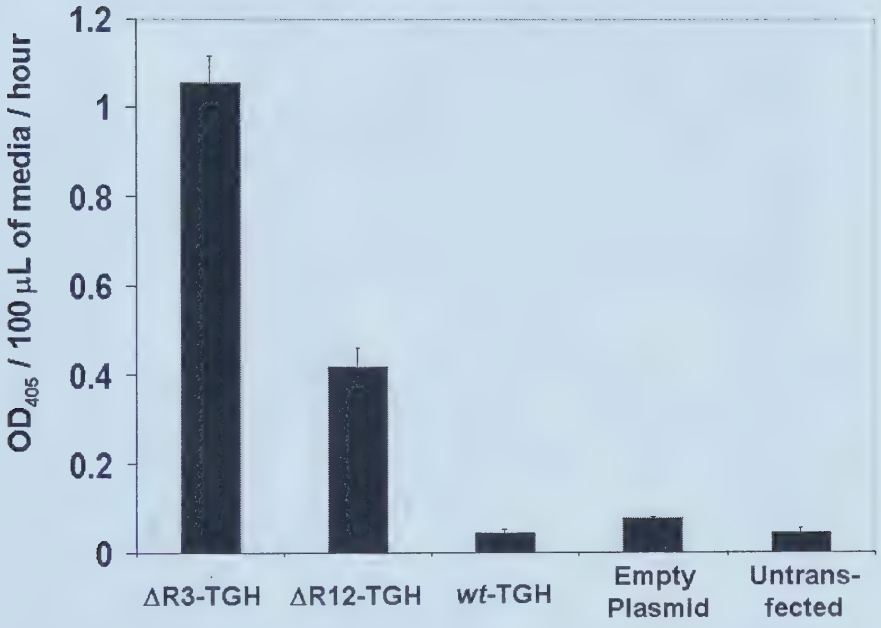


Figure 4-4 ΔR-TGH is catalytically active. Lipase activity in media from McArdle RH7777 cells stably expressing *wt*-TGH, ΔR-TGH, transfected with empty plasmid or untransfected cells after an overnight incubation was determined (*p*-nitrophenyl laurate as substrate). Data represent liberation of *p*-nitrophenol measured spectrophotometrically. Shown are the mean ± SD of triplicate samples and are representative of three independent experiments.

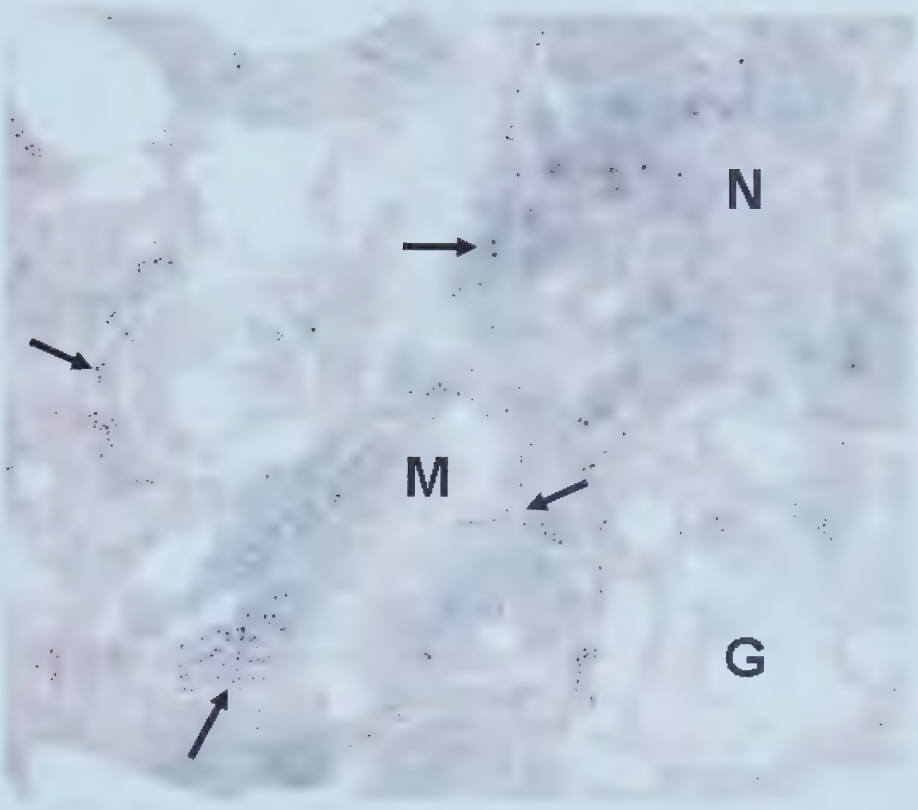


Figure 4-5 Immunogold labeling and electron microscopy of transfected TGH in McArdle RH7777 cells. Immunogold labeling of TGH was performed as described in the Methods. Image is magnified 97,000x. Arrows point to ER elements containing immunogold labeled TGH. N, nucleus; M, mitochondria; G, Golgi.

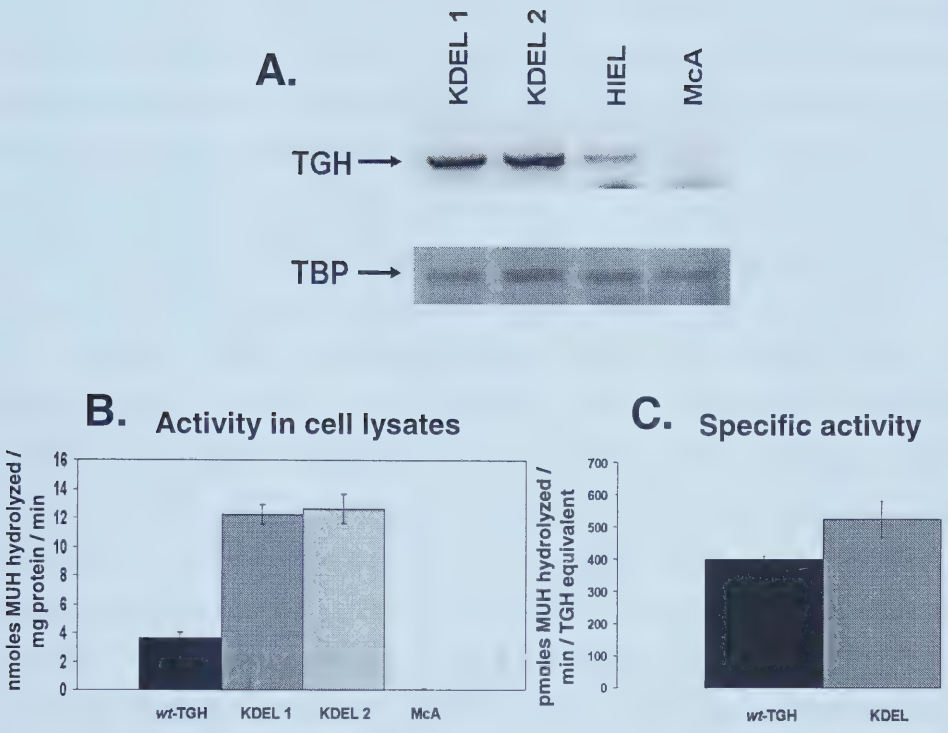


Figure 4-6 Expression of *wt*- and KDEL-TGH in McArdle RH7777 cells.
A. Immunoblots of cell lysates from McArdle RH7777 cells transfected with *wt*-TGH (HIEL), KDEL-TGH (two independent cell lines) or untransfected cells (McA). Immunoblots for TGH show relative levels of this protein; immunoblots for TATA-binding protein (TBP) were performed as a gel loading control.

B. Lipase activity was measured in McArdle RH7777 cell lysates using 4-methylumbelliferyl heptanoate (MUH) as substrate. Data are normalized to amount of cell protein, are the mean ± SD of 6 samples and are representative of three independent experiments.

C. The quantity of *wt*-TGH or KDEL-TGH in cell lysates from stably transfected McArdle RH7777 cells was normalized via immunoblots using fluorescent secondary antibodies. Lysates containing equal amounts of TGH were used in an *in vitro* lipase activity assay with MUH as substrate. Data are the mean ± SD of 3 samples.

expressing *wt*-TGH. *In vitro* lipase activity measurements also showed that the amount of activity in cell sonicates from KDEL-TGH expressing cells was higher than those from *wt*-TGH expressing cells (Figure 4-6 B). Assays using a normalized amount of *wt*-TGH or KDEL-TGH show that the mutation did not reduce the specific *in vitro* catalytic activity of the enzyme (Figure 4-6 C). The enzymatic activity of KDEL-TGH appears to be similar to that of *wt*-TGH since the ratio of KDEL vs. HIEL expression in transfected McArdle cells was calculated to be 4:1 (densitometry of Figure 4-6 A) which agreed well with the observed 4:1 ratio in observed activity (Figure 4-6 B).

McArdle RH7777 cells transfected with *wt*-TGH secrete more robustly lipidated VLDL particles (Lehner and Vance, 1999). The ability of KDEL-TGH to mobilize and secrete intracellular lipids as VLDL was investigated, and it was determined that *wt*-TGH expressing cells are able to secrete more preformed labeled TG and cholesteryl ester (CE) than KDEL-TGH expressing cells during a chase period (Figure 4-7). The amount of lipid synthesis did not differ between cell lines. Hence even with higher levels of intracellular TGH protein and higher *in vitro* lipase activity, the KDEL-TGH expressing cells secreted less VLDL lipids than *wt*-TGH expressing cells. These results suggest that the C-terminal sequence may confer differential access to substrates and may be related to an altered distribution of KDEL-TGH within the ER lumen, however, immunogold electron microscopy of KDEL-TGH in transfected cells showed that the mutant was also localized to the lumen of the ER and some of the staining was also observed in relative proximity to mitochondria (Figure 4-8).

4.3.3 Subcellular localization of wt-TGH and KDEL-TGH

Potential differences in the localization of *wt*-TGH versus KDEL-TGH in transfected cells was further investigated by confocal immunofluorescence microscopy. In order to investigate the localization of KDEL-TGH versus *wt*-TGH concurrently, a plasmid containing both myc-epitope tagged KDEL-TGH and flag-epitope tagged *wt*-TGH was constructed.

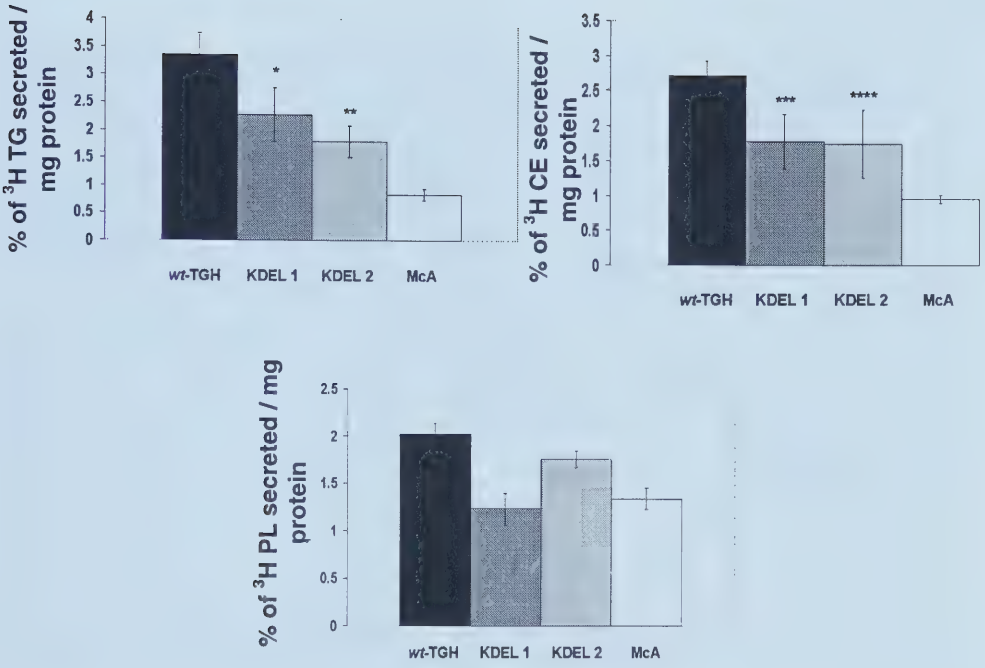


Figure 4-7 Secretion of radio-labeled lipids from McArdle RH7777 cells. Intracellular lipids were labeled with [^{3}H] oleate and secretion of labeled lipids were analyzed after a 4 h incubation. Lipids from both media and cells were extracted and separated by thin layer chromatography. Radioactivity associated with radio-labeled TG, cholesteryl ester (CE), and phospholipids (PL) were measured by scintillation counting. Data represent percent of radio-labeled lipid in media / total (cells plus media) and are normalized to cellular protein levels. Data are mean ± SD of quadruplicate samples and are representative of three independent experiments. For reference, the sum of radio-label measured in cells and media was an average of 220,234 DPM/mg cell protein for TG, 18,040 DPM/mg protein for CE and 260,220 DPM/mg for PL in McArdle cells. * $P \leq 0.016$ ** $P \leq 0.018$ *** $P \leq 0.010$ **** $P \leq 0.022$ with respect to cells transfected with *wt*-TGH.

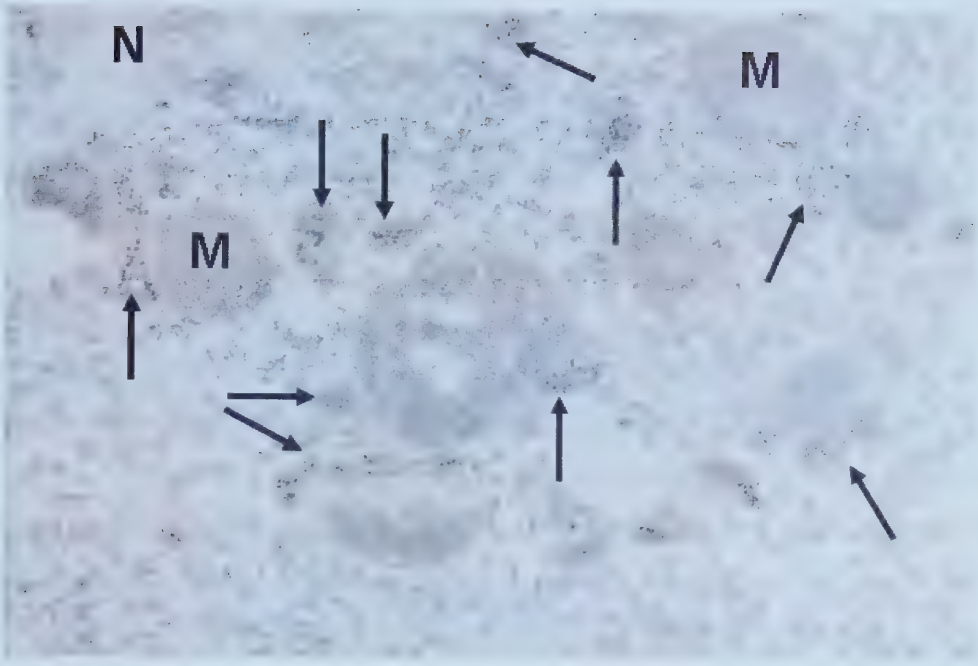


Figure 4-8 Immunogold labeling and electron microscopy of KDEL-TGH in transfected McArdle RH7777 cells. McArdle RH7777 cells stably transfected with KDEL-TGH were analyzed for the localization of TGH by immunogold extectron microscoy. Preparation of cells and immunogold labeling of TGH was performed as described in the Materials and Methods. Image is magnified 45,000x. Arrows point to ER elements containing immunogold labeled TGH. M, mitochondria, N, nucleus.

Our three dimensional model of TGH structure (Alam *et al.*, 2002b) as well as the crystal structure of similar carboxylesterases (Bencharit *et al.*, 2002; Bencharit *et al.*, 2003b) show that the C-terminal tail of TGH is relatively unstructured and therefore an ideal target for inclusion of an epitope tag. The epitope tags were placed immediately N-terminal to either –KDEL or –HIEL. Investigation of these tagged proteins expressed individually verified that inclusion of the tags in this location does not reduce enzyme activity, indicating that tagged TGH is able to fold properly. As expected, the proteins were retained in the ER, and not detected in the culture media. This observation was consistent with previous studies that demonstrated that appending –KDEL or –HIEL to the extreme C-terminus would be both necessary and sufficient to retain these proteins within the ER (Munro and Pelham, 1987; Robbi and Beaufay, 1991). Both epitope tagged TGH constructs were inserted into the pBudCE4.1 plasmid. This vector has two promoters and two multiple cloning sites, facilitating stable transfection of McArdle RH7777 cells without biases related to the transfection of the two constructs. The KDEL-TGH and *wt*-TGH were immunolocalized by confocal microscopy using antibodies directed against the epitope tags. As shown in Figure 4-9 A, substantial staining of *wt*-TGH is present in a peripheral region in the cell that is devoid of KDEL-TGH. In order to verify that KDEL-TGH and *wt*-TGH reside in different regions of the ER, confocal immunofluorescence experiments were performed with cells that were expressing KDEL-TGH or *wt*-TGH individually and without epitope tags. Colocalizations were performed with the KDEL-bearing and ER resident protein BiP (also know as Grp78, (Munro and Pelham, 1987)). Colocalizations with KDEL-TGH and BiP show nearly complete overlap, whereas the overlap is far less extensive between *wt*-TGH and BiP (Figure 4-9 B), supporting the concept that *wt*-TGH is not found in the same regions of the endoplasmic reticulum as other KDEL-bearing proteins. The staining for *wt*-TGH also appears to be more peripheral than for BiP, which is where the majority of lipid storage droplets are found. Another difference between KDEL and *wt*-TGH that can be discerned from these images is that KDEL-TGH is found in a diffuse pattern, whereas *wt*-TGH is in a lattice pattern.

A.

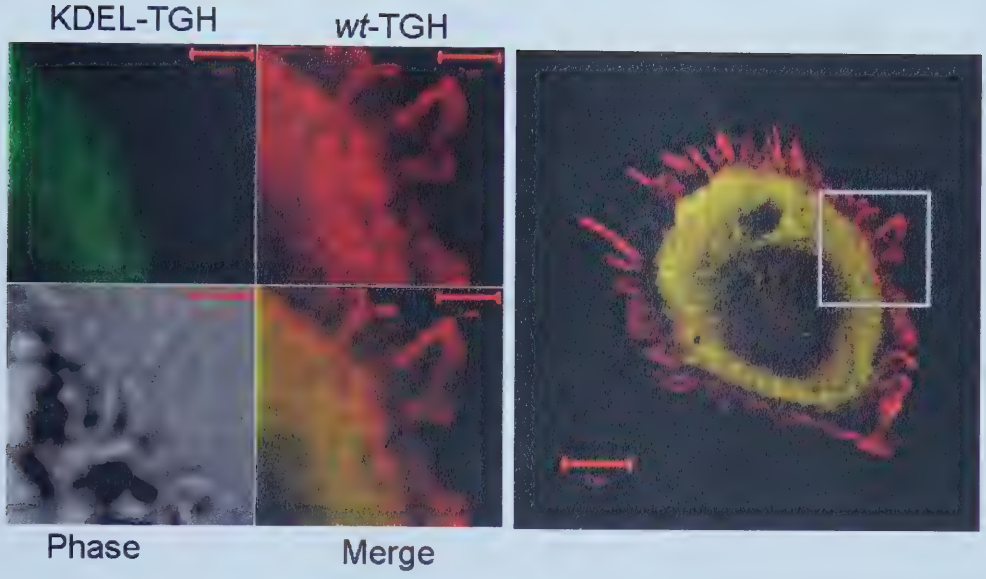
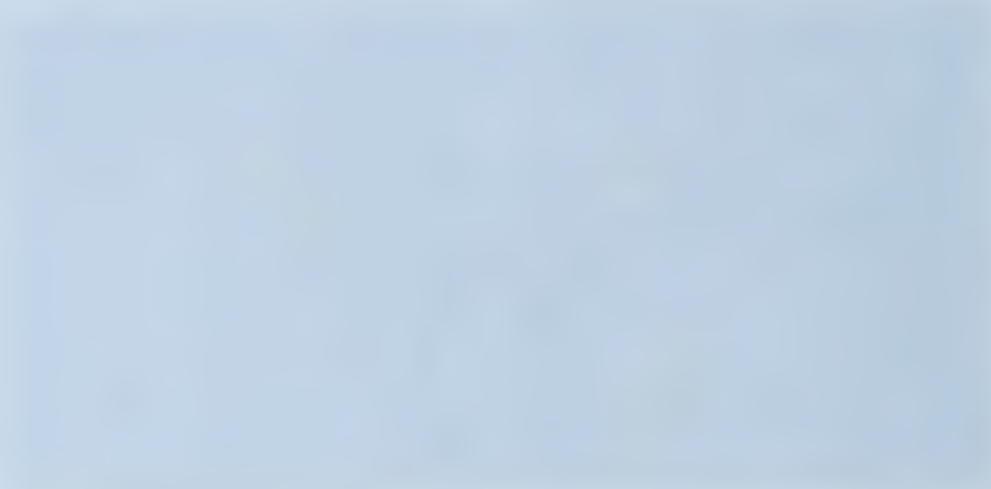


Figure 4-9 Confocal immunofluorescence microscopy of *wt*-TGH and KDEL-TGH.

A. McArdle RH7777 cells stably expressing flag-tagged *wt*-TGH and myc-tagged KDEL-TGH were fixed onto microscope cover slips and decorated with anti-flag and anti-myc antibodies as described in Materials and Methods. The flag-tagged *wt*-TGH and myc-tagged KDEL-TGH were visualized with secondary anti-mouse and anti-rabbit antibodies conjugated to Texas Red and fluorescein or Alexa488 respectively. Images were obtained using a confocal microscope. The boxed region is shown under higher magnification. Bars represent 2 μm for higher magnification (left); 5 μm for lower magnification (right).



B.

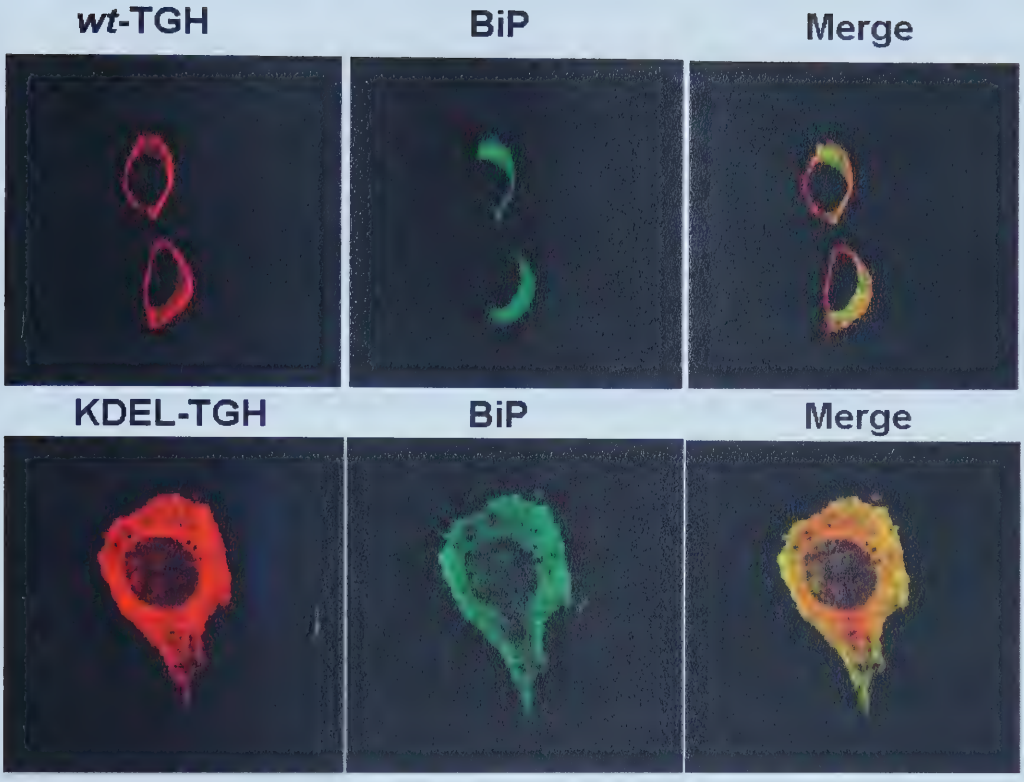


Figure 4-9 Confocal immunofluorescence microscopy of *wt*-TGH and KDEL-TGH.

B. McArdle RH7777 cells stably expressing *wt*-TGH or KDEL-TGH without epitope tags were fixed onto microscope cover slips as in Figure 4-9 A. Incubations were performed with a monoclonal mouse anti-BiP antibody and polyclonal rabbit anti-TGH antibodies, which were stained with secondary anti-mouse and anti-rabbit antibodies conjugated to fluorescein and Texas Red respectively. Images were obtained on a confocal microscope as they were in A. This figure was developed in cooperation with Wenhui Gao.

4.3.4 wt-TGH but not KDEL-TGH is enriched in regions containing neutral lipid droplets

After observing differences in the localization of KDEL-TGH versus *wt*-TGH with respect to lipid droplets, transfected McArdle RH7777 cells that had been incubated with or without oleate for 4 h were stained with Nile Red to observe neutral lipid droplets, while simultaneously observing KDEL-TGH or *wt*-TGH via their epitope tags. The images in Figure 4-10 A and B show that the majority of lipid droplets are found in the periphery of the cell where *wt*-TGH is more highly concentrated than KDEL-TGH. Therefore, increased ability of *wt*-TGH to mobilize lipids for secretion on VLDL may be explained by the differences observed in localization and association with lipid droplets. Association with lipid droplets may also explain why TGH co-fractionated with ER elements and lipid droplets in previous studies (Lehner and Vance, 1999).

Cells that were transfected with empty pBudCE4.1 plasmid were used in control experiments that demonstrate staining for both epitope tags was specific. Immunofluorescence was not observed in cells transfected with empty plasmid using either the anti-myc or anti-flag antibodies. Transfection of *wt*-TGH or any of the mutants described did not recognizably change the morphology of cells. McArdle RH7777 cells, however, display diversity in morphology amongst cells on a single dish and in response to culture conditions.

4.4 Discussion and conclusions

Three dimensional modeling of TGH based on the acetylcholine esterase crystal structure suggests that TGH contains two disulfide bridges and a free cysteine residue ((Alam *et al.*, 2002b) and Figure 1-9). Apparently the ER environment is essential for TGH to achieve the correctly folded state as attempts at expression in *E. coli* have resulted in production of an inactive protein (Alam *et al.*, 2002a). Further, expression of TGH in cytosol by removal of the amino-terminal signal sequence has resulted in an inactive protein (personal communication from collaborators at GlaxoSmithKline).

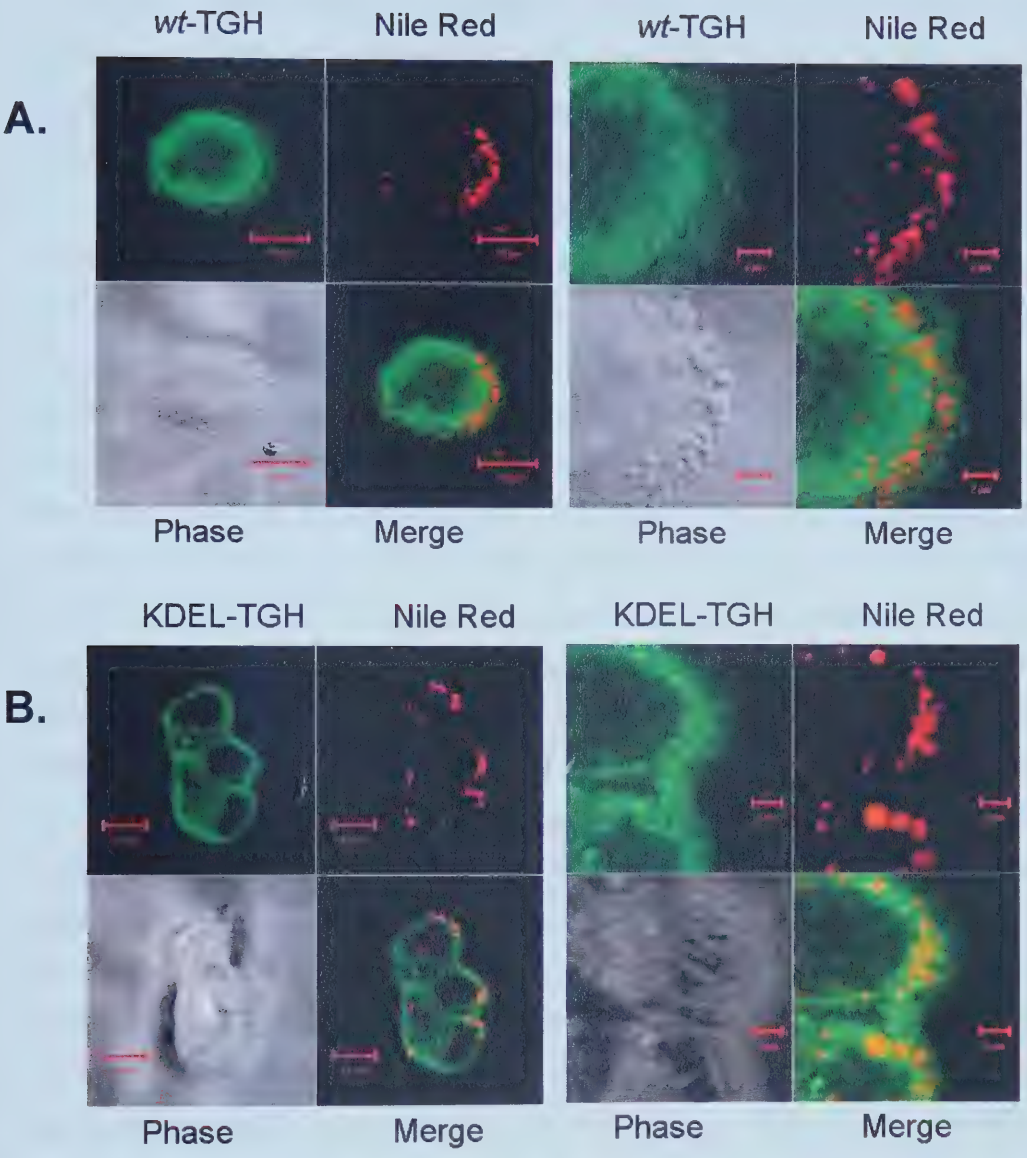


Figure 4-10 Colocalization of *wt*-TGH or KDEL-TGH with lipid droplets. **A-B.** Images were prepared as they were in Figure 4-9, except cells were incubated 4 h with 4 mM oleate / 0.5% BSA prior to fixation and staining. Nile Red was used to stain neutral lipid droplets and a monoclonal anti-flag antibody was used to detect flag epitope tagged *wt*-TGH (**A.**) or polyclonal rabbit anti-myc antibodies utilized to detect myc epitope tagged KDEL-TGH (**B.**). Images on the right are at higher magnification. Bars represent 10 μm for lower magnification (left); 2 μm for higher magnification (right).

It is likely that the ER environment provides the necessary disulfide isomerases and folding chaperones to facilitate the appropriate folding state of this protein. Apparently once this is achieved, TGH is stable enough to exit the ER without compromising specific enzyme activity.

The subcellular localization of TGH may not only be vital for folding, but also to direct the fate of fatty acids released by TGH-catalyzed lipolysis. As indicated previously (Section 1.3.6), expression of the cytosolic lipase HSL in hepatoma cells resulted in oxidation of the liberated fatty acids rather than secretion as seen with TGH. Our finding that the carboxy-terminal sequence of TGH is responsible for its retention in the ER is not completely novel, as this has been demonstrated for other soluble ER proteins. For example, deletion of the C-terminal six amino acids from rabbit and human Es 22, which is very similar to TGH, and baculovirus mediated expression in Sf 21 cells resulted in a secreted protein that could be purified from media for crystallization (Bencharit *et al.*, 2002; Bencharit *et al.*, 2003b). The data presented here confirm the C-terminal sequence is the primary element that keeps TGH in the ER. Development of the ΔR-TGH expression system also provided a valuable tool for future studies, as a partially purified TGH protein in the cell culture media (versus a cell homogenate) was readily obtained without compromising specific enzyme activity. This system was employed in Chapters 5 and 6 of this Thesis where substrate specificity and post-translational modification of TGH are investigated. Further, media containing ΔR-TGH is now routinely used as a positive control in the fluorescent lipase assay (MUH as substrate) in our laboratory. These data are also the foundation for developing a scheme for expressing and purifying mouse TGH for future studies described in Chapter 8. Clearly this system has contributed a valuable tool for our laboratory and to our understanding of TGH.

The C-terminal sequence deleted from TGH in order to produce the ΔR-TGH secreted enzyme may be functionally relevant in directing TGH to a subdomain of the ER. In order to explore this possibility, a mutant of TGH that carries the –KDEL C-terminal sequence rather than the endogenous –HIEL sequence was created. Several assessments were then made, including the

ability of expressed TGH to mobilize intracellular lipids in transfected cells and to colocalize with other soluble proteins in the ER lumen as well as with neutral lipid droplets. Because *wt*-TGH expressing cells secreted more neutral lipid than KDEL-TGH expressing cells, we speculated that this C-terminal sequence not only confers retention in the ER, but also concentrates this protein in a microdomain of this organelle where TGH may more readily access its substrate or where the majority of VLDL assembly occurs, allowing more efficient secretion of lipids on apoB. This hypothesis is supported by our observation of non-uniform cellular distribution of *wt*- and KDEL- TGH in ER elements. Confocal immunofluorescence analyses showed *wt*-TGH in unidentified projections near the cell surface that contained little or no KDEL-TGH. The increased ability of *wt*-TGH to mobilize lipid droplets for VLDL assembly compared to KDEL-TGH may be attributed to better access to this substrate since the majority of lipid droplets are localized near the periphery of cells.

It is established that the ER is a diverse environment with specialized activities sequestered in particular regions. Examples include regions for ribosome attachment (i.e. smooth versus rough ER), regions for vesicle docking and budding and regions surrounding the nucleus that contain nuclear pores. It is presently unclear how proteins involved in different activities are delivered to these specific regions of the ER. Similarly, it is feasible that a region of the ER exists that is specialized in lipoprotein assembly. The mechanism of how TGH is delivered to this region remains uncertain, but appears to involve the C-terminal -HIEL sequence. To our knowledge, this is the first time a putative sequence culminating in specific trafficking of proteins within the ER has been identified. Potentially a receptor other than the well-characterized KDEL receptor (Lewis and Pelham, 1990; Tang *et al.*, 1993; Wilson *et al.*, 1993) is involved in retrieval of soluble proteins bearing C-terminal sequences like -HIEL from the Golgi apparatus and delivers them to this location of the ER. Alternately, TGH may associate with proteins within the ER that sequester it to regions in proximity to lipid droplets or regions involved in lipoprotein assembly. The latter mechanism would require an interaction dependent on the C-terminal sequence, or it would

also function for the KDEL-TGH mutant. It has been proposed that the exit of the assembled VLDL particle from the ER may also require specialized machinery as VLDLs are much larger than transport vesicles (Schekman and Mellman, 1997). This machinery may also be localized to this region of VLDL assembly by the same mechanism as TGH.

CHAPTER 5:

Analysis of apoB associated lipids as a substrate pool for TGH

The majority of the information presented in this Chapter has been published in Gilham, D., M. Alam, W. Gao, D.E. Vance, and R. Lehner. (2005) Triacylglycerol hydrolase is localized to the endoplasmic reticulum by an unusual retrieval sequence where it participates in VLDL assembly without utilizing VLDL lipids as substrates. *Mol. Biol. Cell.* 16:984-996, and is adapted into this Thesis with permission

5.1 Introduction

There is consensus that VLDL assembly at least partially (if not completely) occurs in the ER. Therefore, it was examined whether neutral lipids on apoB containing particles could be included in the TGH substrate pool as they will co-exist in the ER lumen. As discussed in Chapter 1, not all newly made apoB is assembled into VLDL and the partially lipidated, secretion-incompetent apoB undergoes degradation. This process likely occurs following retrotranslocation into cytosol, where protein degradation is mediated by the proteasomal system. During the process of apoB degradation, lipids associated with this apolipoprotein need to be removed or hydrolyzed. Potentially, TGH may be involved in the "delipidation" process of misfolded apoB containing particles. Experiments described in this Chapter were designed to address whether TGH can hydrolyze TG in fully folded and secreted apoB lipoproteins or TG associated with the primordial apoB containing particle present in the ER.

5.2 Materials and Materials

5.2.1 Reagents

Polyclonal anti-apoB antibodies were purchased from Chemicon International, Temecula, CA. Prolong Antifade and fluorescently labeled secondary antibodies were purchased from Molecular Probes, Eugene, OR. NADH and *tert*-butyl hydroperoxide were from Sigma-Aldrich, Oakville, ON, Canada. [9,10(n)-^{3}H] Oleic acid (10 Ci/mmol) was obtained from Amersham-Pharmacia Canada (Oakville, ON, Canada).

5.2.2 Colocalization of apoB with TGH

McArdle RH7777 cells stably expressing TGH were grown on microscope coverslips, fixed and prepared for confocal microscopy as described in Section 4.2.10. Primary antibodies used were anti-TGH and anti-apoB (both 1:500 dilution). Secondary antibodies were conjugated to Texas Red (for TGH) and FITC (for apoB). Confocal images were obtained as in Section 4.2.10.

5.2.3 Cross-linking of TGH

Primary rat hepatocytes were isolated as described in Section 2.5.2, and from these preparations, 1.5 x 10^6 cells were plated on 60 mm diameter dishes. Cells were allowed to settle and attach to 60 mm diameter collagen coated dishes for 5 hours. Media was changed to DMEM ± 0.4 mM oleate complexed to 0.5 % BSA for 4 h. Cells were washed with PBS, then collected by scraping into 2 mL of ice cold PBS. Cells were lysed using 15 stokes of a Potter-Elvehjem tissue homogenizer. Microsomes were prepared by centrifugation as described in Section 3.2.2 and resuspended in PBS. Microsomes derived from both treatment groups (± oleate) were divided into 4 aliquots of equal volume. Protein cross-linking was performed using dithiobis[succinimidylpropionate] (DSP) or dimethyl 3,3´-dithiobispropionimidate•2 HCl (DTBP; Pierce Biotechnology, Brockville, Ontario, Canada). These agents were added from concentrated solutions prepared immediately before use and delivered to a final concentration of 4 mM. Both of these reagents are cleavable with reducing agents, are homobifunctional, have similarly sized spacer arms and permeate biological membranes. After 40 min the cross-linking reactions were quenched by adjusting the incubation solution to contain 100 mM Tris using a 1 M stock pH 8.0. Samples were allowed to sit on ice for 10 min. Triton X-100 was then added to a final concentration of 1%. The samples were then incubated with 25 µL of protein-A Sepharose beads for 2 hours at 4 °C on a rotating rack to clear species that may bind non-specifically to the beads. Following centrifugation, supernatants were incubated with 10 µL of anti-human apoB antibody (Roche Diagnostics, Mannheim, Germany) on a rotating rack for 12 h at 4°C, then 35 µL of protein-A Sepharose was added and the mixture was incubated for an additional 4 h. The beads were pelleted by brief centrifugation, then washed twice with excess buffer containing 50 mM Tris pH 8, 150 mM NaCl, 0.5 % Triton X-100 and 0.1 % SDS. Finally, beads were washed once with PBS. Beads were resuspended in denaturing electrophoresis sample treatment buffer, boiled for 5 min, then the proteins were resolved by SDS-PAGE (10 %). Samples were

transferred to nitrocellulose membranes and probed for the presence of TGH via immunoblot using anti-porcine TGH polyclonal antibodies.

5.2.4 Preparation of secreted [^{3}H] oleate-labeled lipoproteins from McArdle RH7777 cells

McArdle RH7777 cells were grown to ~80% confluence in 60 mm diameter dishes. Media was changed to 2 mL of DMEM containing 0.4 mM oleate, 0.5% BSA and 5 μCi [^{3}H] oleate for 2.5 h. Following this loading period, cells were washed three times for 10 min in DMEM containing 0.5% BSA to remove fatty acids non-specifically associated with the exterior of cells. The cells were then incubated 4 h in DMEM. Aliquots of this media containing labeled lipoproteins were transferred onto McArdle RH7777 cells stably transfected with ΔR-TGH, or non-transfected cells ± 1.2 units (U) of lipoprotein lipase (LpL) from *Chromobacterium viscosum* (Sigma-Aldrich). (One unit is defined as the amount of enzyme that liberates 1 μmol of oleate from trioleoylglycerol per min at pH 8.0 and 40°C.) One milliliter of media from each incubation was collected after 14 h and lipids extracted, separated by TLC and analyzed by scintillation counting as above. Aliquots of media from dishes of ΔR-TGH or non-transfected cells ± 0.6, 1.2 and 2.5 U LpL were also analyzed for lipase activity.

5.2.5 Preparation of intracellular [^{3}H] oleate-labeled apoB containing particles from rat hepatocytes

In order to assess lipids associated with intracellular apoB containing particles as a substrate for TGH, hepatocytes were isolated by collagenase perfusion of the liver from male Sprague-Dawley rats (body weight ~150 g) fed *ad libitum* as described in Section 2.5.2. Hepatocytes were plated on 100 mm collagen coated cell culture dishes in DMEM containing 15% FBS and allowed to settle and attach to the dishes for 5 h. Media were changed and incubation continued for 14 h. Media was then replaced with DMEM containing 0.4 mM oleate, 0.5% BSA and 8 μCi/mL [^{3}H] oleate for 4 h. Cells were washed twice with PBS and scraped into buffer containing 50 mM Tris pH 7.4, 1 mM EDTA, and

250 mM sucrose. Cells were sonicated briefly, then microsomes prepared as in Section 3.2.2. Microsomes were resuspended in 0.2 M sodium carbonate pH 12 and placed on ice for 15 min. The suspension was then centrifuged at 350,000 x*g* for 45 min. at 4°C. The supernatants, containing microsomal lumenal apoB containing particles`, were transferred to new tubes, and the pH adjusted to 7.4 with 1 M Tris pH 7.4. ApoB containing particles were isolated from the supernatants by immunoprecipitation with anti-apoB antibodies bound to protein A-Sepharose beads. Beads carrying apoB containing particles were washed twice with PBS, then divided into 10 equal aliquots. One aliquot was kept at 4°C until analysis of all other samples. Three aliquots received DMEM that did not contain lipases, three received DMEM containing ΔR-TGH, and three received an equal volume of DMEM containing LpL with the same *in vitro* lipolytic activity with MUH as those with ΔR-TGH. Samples were incubated for 14 h at 37°C while rotating end-over-end. All samples were then treated with chloroform/methanol 2:1 and lipids extracted via recovery of the organic phase. The lipids were then separated via TLC and radioactivity associated with different lipid species determined by scintillation counting as described in Section 2.8.

5.2.6 Assessing cell integrity by lactate dehydrogenase (LDH) activity assays

Assays of LDH activity were employed to determine if extracellular lipases encountered by McArdle RH7777 cells disrupted cell integrity. LDH activity in both cell lysates and media of McArdle RH7777 cells encountering extracellular lipase activity was determined essentially as described in Section 3.2.11 for primary rat hepatocytes incubated with lipase inhibitors. Modifications from Section 3.2.11 were required to adapt to this context. Briefly, McArdle RH7777 cells (untransfected or ΔR-TGH expressing) were plated on 60 mm dishes and incubated overnight. Media were removed and replaced with serum free DMEM ± 1.2 or 2.5 U LpL for 14 h. Some cells were incubated for 2 h with 2 mL of DMEM containing 400 μM *tert*-butyl hydroperoxide (a compound known to induce cell disruption). Samples of media were collected after incubation periods and cleared of cellular debris by centrifugation at 2,600 x*g* for 2 min. Cells were

scraped into 1 mL of PBS containing Complete® protease inhibitors and briefly sonicated. Assays were conducted in triplicate in clear 96 well microtitre plates using the volumes, substrate solution and time course applied in Section 3.2.11. LDH activity is presented as the percent of LDH activity in the media relative to total (cells plus media) after 3 min of reaction time.

5.3 Results

5.3.1 ApoB and TGH partially colocalize and can be chemically cross-linked

It has been hypothesized that TGH mobilizes TGs for VLDL assembly from droplets associated with the ER (Figure 1-12). Since VLDL assembly also occurs in the ER, the TGH substrate pool could potentially also include lipids already loaded onto an apoB containing particle, or assembled VLDL before secretion. To explore the proximity of TGH to apoB, confocal immunofluorescence microscopy was used to examine a colocalization pattern. As shown in Figure 5-1 A, a substantial overlap in the staining pattern of these two proteins was observed.

An interaction between apoB and TGH has been previously suggested using a proteomic approach to identify all proteins that interact with apoB (Rashid *et al.*, 2002). In order to confirm results and support the proposed interaction between apoB and TGH, the cross-linking reagents DTBP and DSP were employed. Following cross-linking with DTPB, an antibody developed against apoB could be used to immuno-isolate TGH (Figure 5-1 B). The interaction was stronger when cells were not loaded with oleate prior to cross-linking, however, the methods employed were not purely quantitative. Therefore, the differences observed with or without oleate loading of cells remains speculative. Isolation of TGH with apoB antibodies could not be accomplished using DSP as the cross-linking agent.

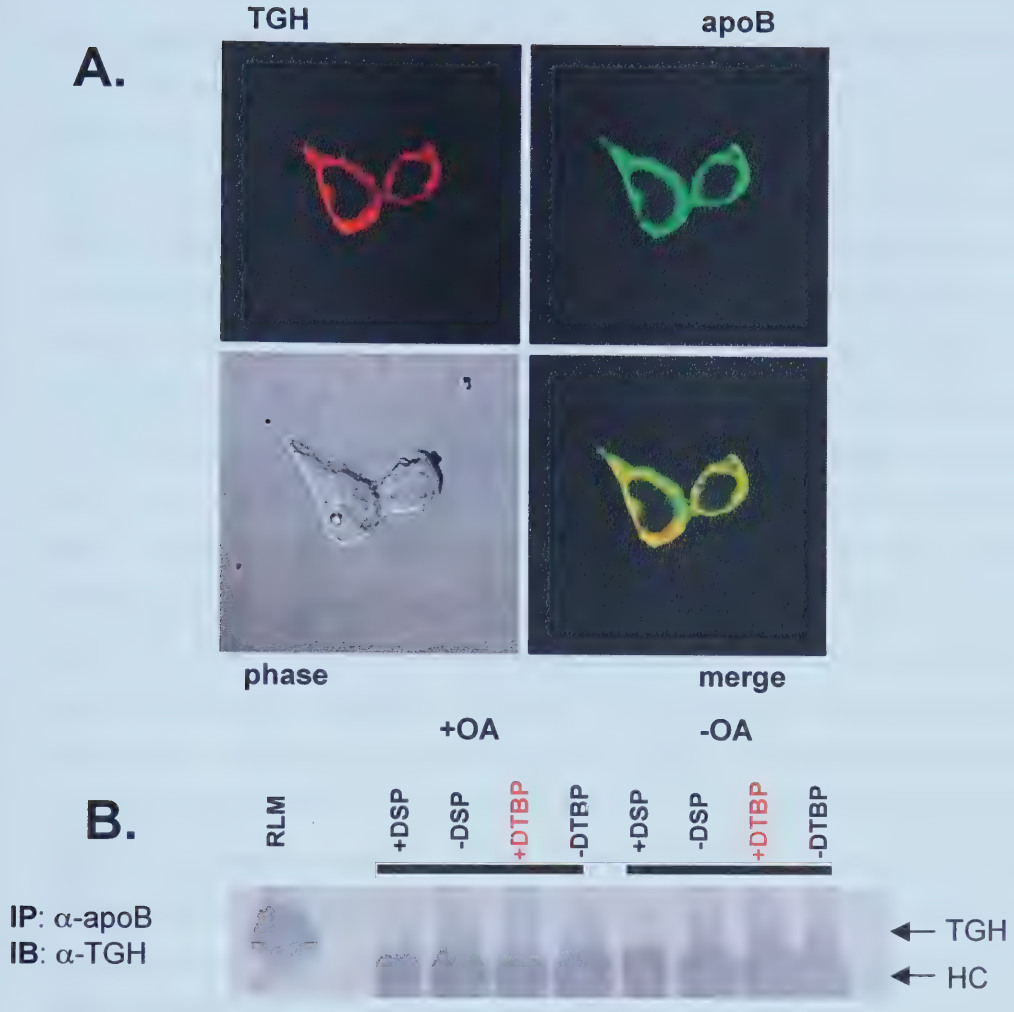


Figure 5-1 TGH co-localizes with and can be cross-linked to apoB.
A. McArdle RH7777 cells stably expressing TGH were grown on microscope cover slips and fixed. TGH and apoB were decorated with Texas Red and fluorescein, respectively, using immunostaining procedures described in Materials and Methods. Images were obtained using a confocal microscope. This image was provided by Wenhui Gao.

B. Rat hepatocytes were incubated in DMEM ± 400 μM oleic acid (OA) for 4 hours. Cells were harvested and microsomes prepared. Microsomes were incubated with the cross-linking agents DSP or DTBP. After quenching these agents, microsomes were solubilized with detergent and apoB isolated by immunoprecipitation (IP). Samples were subjected to SDS-PAGE, and immunoblotting (IB) was used to probe for TGH that had co-isolated with apoB. An aliquot of rat liver microsomes (RLM) was used to identify the TGH band. Also indicated is the heavy chain (HC) of the antibody used for IP.

5.3.2 Lipids associated with secreted lipoproteins are not substrates for TGH

The demonstration that TGH and apoB are in close enough proximity to each other to engage in a potential interaction, there is validation to the concept that TGH may hydrolyze apoB associated lipids. To determine if apoB containing particles provide substrates for TGH, [^{3}H] oleate labeled lipoproteins secreted from McArdle RH7777 cells were generated, then transferred to cells that secrete ΔR-TGH in order to assess the activity of TGH towards apoB associated lipids. If TGH hydrolyzed VLDL lipids, a time-dependent decrease of radio-labeled lipids from the media would be observed due to uptake of the lipolytic products (fatty acids). As a control, microbial LpL was added exogenously to untransfected cells. LpL is known to hydrolyze TGs on VLDL and other apoB containing particles such as chylomicrons and LDL (Sugiura and Isobe, 1974). As shown in Figure 5-2 A, there was substantial lipase activity due to secreted TGH in the media of ΔR-TGH transfected cells after 14 h, exceeding the activity seen with 2.5 U of LpL. The amount of labeled TG and CE found in the media, however, were not different between untransfected cells and ΔR-TGH expressing cells after 14 h incubation with labeled lipoproteins (Figure 5-2 B and C). In contrast, incubations that included LpL had markedly reduced amounts of labeled TG and CE in the media. The results indicate that ΔR-TGH does not hydrolyze TG or CE associated with fully assembled secreted VLDL.

Potentially, the build up of lipase activity resulting from ΔR-TGH secretion or the addition of exogenous LpL disrupted McArdle RH7777 cells over the course of our experiments. To test this, LDH assays were used to probe for leakage of this normally cytosolic enzyme into the culture media. The results of these assays (Figure 5-3) indicate the presence of these extracellular lipases do not disrupt the integrity of the cells.

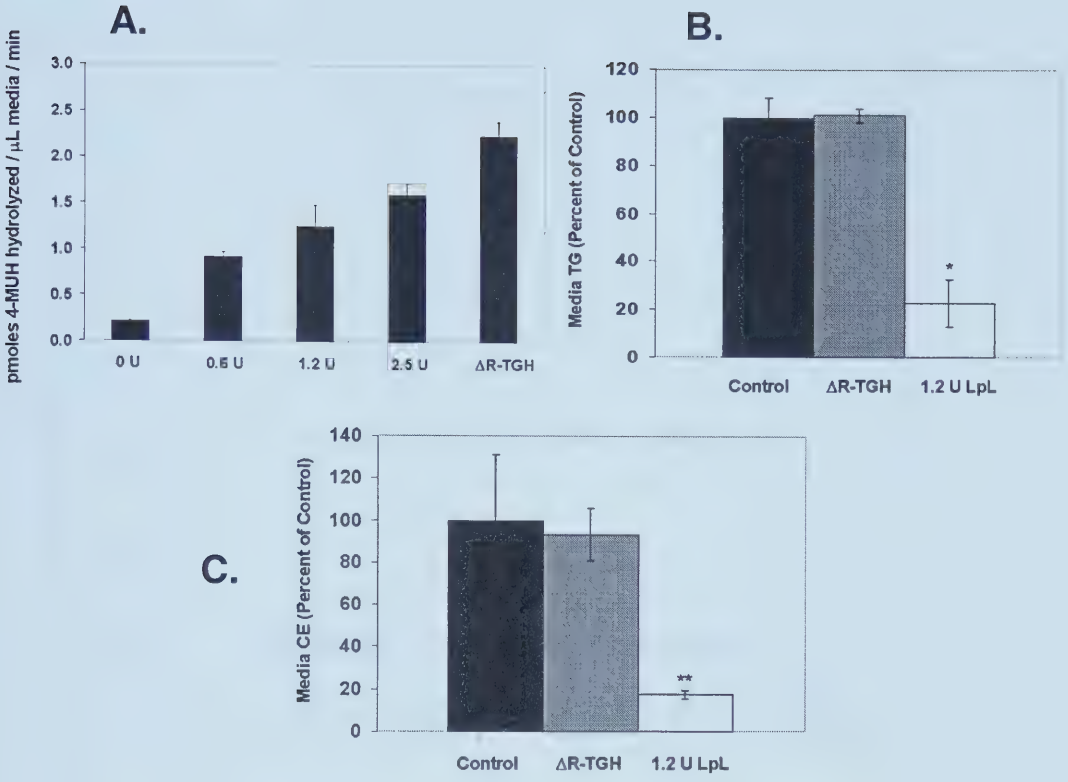


Figure 5-2 TGH does not utilize lipids from secreted lipoproteins. **A.** Lipase activity was assayed in media of McArdle RH7777 cells stably transfected with ΔR-TGH or untransfected McArdle RH7777 cells ± microbial lipoprotein lipase (LpL; number of enzymatic units (U) are indicated) after a 14 h incubation. 4-Methylumbelliferyl heptanoate (MUH) was used as a substrate. Data are the mean ± SD of 6 samples and are representative of three independent experiments. **B,C.** Media containing radio-labeled lipoproteins were collected from McArdle RH7777 cells as described in Materials and Methods. The amount of radio-labeled triacylglycerol (TG; **B.**) and cholesteryl ester (CE; **C**) remaining in media containing labeled lipoproteins was assessed after a 14 h incubation on untransfected McArdle RH7777 cells (control), untransfected McArdle RH7777 cells supplemented with 1.2 U LpL or on McArdle RH7777 cells stably transfected with ΔR-TGH. For reference, the radio-label associated with TG in the media of control cells after the 14 h incubation was an average of 7415 DPM and 2570 DPM for CE. **Figure 6 B:** *$P \leq 0.0006$ **Figure 6 C:** **$P \leq 0.044$ with respect to control.

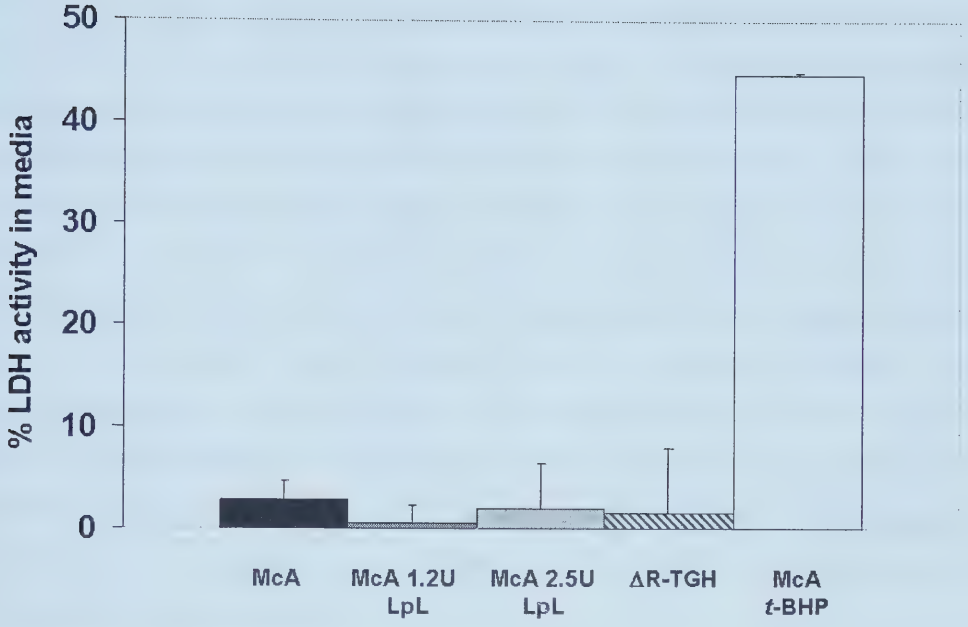


Figure 5-3 Lipases present in culture media do not disrupt McArdle RH7777 cell integrity. Lactate dehydrogenase (LDH) activity was assayed in both cells and media of McArdle RH7777 cells (untransfected or stably expressing ΔR-TGH) after 14 h incubation in DMEM ± 1.2 or 2.5 U of microbial lipoprotein lipase (LpL). LDH activity is represented as the percent of activity in media relative to total (cells and media). To induce cell damage and LDH leakage, some cells were incubated for 2 h with 400 μM *tert*-butyl hydroperoxide (*t*-BHP).

5.3.3 Lipids associated with intracellular apoB containing particles are not substrates for TGH

Intracellular apoB containing entities with various folding and lipidation states will develop along with the assembly of secretion competent particles (Fisher and Ginsberg, 2002; Olofsson *et al.*, 1999). De-lipidation of misfolded or inadequately lipidated particles may be required for retrotranslocation of apoB for degradation by the cytosolic proteasome system (Olofsson *et al.*, 1999; Yao *et al.*, 1997). TGH may recognize these secretion-incompetent states and facilitate lipid removal. In order to assess the ability of TGH to hydrolyze radio-labeled lipids from intracellular apoB, primary rat hepatocytes were incubated with [^{3}H] oleate, microsomes were isolated, apoB containing particles were immunoprecipitated and then these were incubated with TGH and LpL. The amount of lipase activity of TGH or LpL was normalized in assays using MUH as substrate. As shown in Figure 5-4, TGH was not able to hydrolyze lipids associated with intracellular apoB, while LpL dramatically reduced levels of TG and liberated free fatty acids.

5.4 Discussion

ApoB is co-translationally lipidated in the ER to form a primordial entity that matures into a VLDL particle (Olofsson *et al.*, 1999; Shelness and Sellers, 2001). Here it is shown that apoB-associated lipids are not a substrate for TGH. This was unexpected as apoB and TGH can be cross-linked in microsomes isolated from primary rat hepatocytes, indicating a possible interaction between these two proteins (Figure 6-4 B; (Rashid *et al.*, 2002)). In addition, partial colocalization between apoB and TGH has been observed in confocal images taken from TGH transfected McArdle RH7777 cells, and this suggests their co-existence in the same compartment during initial apoB containing lipoprotein assembly (Figure 6-4 A). Colocalization with TGH likely reflects apoB that is being assembled into VLDL, while apoB that does not colocalize may represent apoB that is being synthesized on the rough ER, has entered the post-ER secretory pathway or is engaged in endocytosis.

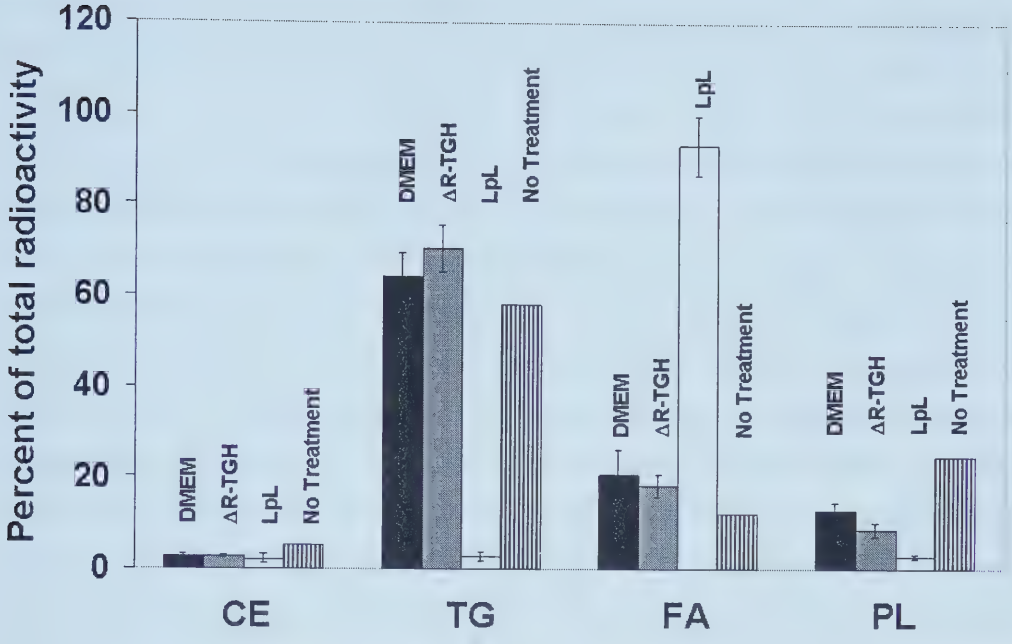


Figure 5-4 TGH does not hydrolyze lipids on intracellular apoB containing particles.
Lipids in primary rat hepatocytes were labeled by incubation with [^{3}H] oleate. Microsomes were prepared and carbonate extracted to release lumenal contents and peripheral membrane proteins. Liberated apoB containing particles were isolated by immunoprecipiation, then incubated for 14 h with ΔR-TGH or microbial lipoprotein lipase (LpL) containing an equal amount of *in vitro* lipase activity. Following this incubation, lipids were extracted, separated by TLC and radioactivity associated with each lipid species determined by scintillation counting. Shown are radioactivities associated with cholesteryl ester (CE), triacylglycerol (TG), free fatty acids (FA) and phospholipids (PL). For reference, the radio-label associated with TG in control incubations after 14 h was an average of 2126 DPM. Data are the mean ± SD of 3 independent samples.

Previous studies have shown that TGH activity enhances VLDL lipidation in transfected hepatoma cell lines (Lehner and Vance, 1999), also signifying that apoB associated TG is not a substrate for TGH within the ER. Furthermore, inhibition of TGH activity did not lead to the accumulation of intracellular apoB (Chapter 3) which also suggests that the role of TGH is not delipidation of misfolded apoB. It is possible that TGH requires appropriate lipid droplet coat proteins to facilitate its interaction with a lipid substrate. Potentially apoB is not a compatible coat protein and may have a barrier function toward TGH-mediated lipolysis. Other lipid droplet coat protein(s) in the ER lumen of hepatocytes, such as apolipoproteins, may modulate or regulate TGH access to its endogenous substrate pools. Evidence that TGH interacts with other proteins in the ER is presented in Chapter 7, however, TGH binding partners have not been conclusively identified to date. Importantly, the data within the current Chapter present a mechanism for vectored movement of TG from storage depots towards VLDL assembly as once associated with apoB, TGs are protected from lipolysis by this enzyme.

Chapter 6:

Post-translational modifications of TGH

A) Glycosylation

Some of the information presented in this Chapter has been published in Gilham, D., M. Alam, W. Gao, D.E. Vance, and R. Lehner. (2005) Triacylglycerol hydrolase is localized to the endoplasmic reticulum by an unusual retrieval sequence where it participates in VLDL assembly without utilizing VLDL lipids as substrates. *Mol. Biol. Cell.* **16**:984-996, and is adapted into this Thesis with permission

6.1 Introduction

TGH has been shown to be a glycoprotein via recombinant expression in Sf-9 cells (Alam *et al.*, 2002b). A lone glycosylation site is predicted at Asn 79, and the crystal structure of a similar carboxylesterase from rabbit in a fully glycosylated state has been reported (Bencharit *et al.*, 2002). The crystal structure showed *N*-linked glycosylation on this residue, which is likely conserved in TGH. The requirement of glycosylation for catalytic activity was characterized, as glycosylation is known to mediate interaction with chaperones found in the ER that facilitate protein folding.

6.2 Materials and Methods

6.2.1 Reagents

Tunicamycin, was from Sigma-Aldrich, Oakville, ON, Canada. The TGH specific inhibitor GR148672X (Chapter 3) was from GlaxoSmithKline (Les Ulis, France).

6.2.2 Tunicamycin treatment

The secreted mutant of TGH (ΔR-TGH) introduced in Chapter 4 was utilized to investigate the requirement of glycosylation for TGH to achieve proper folding and enzymatic activity. Recall ΔR-TGH lacks the C-terminal 4 amino acids, resulting in secretion of the enzyme into the culture medium in a catalytically active state. In 100 mm diameter dishes, 4.5×10^6 McArdle RH7777 cells stably expressing ΔR-TGH were grown overnight. Cells were subsequently incubated for 1 h in DMEM containing 10% FBS, 10% HS and ± 5 μg/mL tunicamycin. Cells were washed with PBS and incubated for 5 h in serum free DMEM ± 5 μg/mL tunicamycin. Media (7 mL) were collected and centrifuged at 600 x*g* for 2 min to pellet cell debris. Cells were scraped into ice-cold PBS and sonicated briefly. The amount of protein in these lysates was determined as described in Chapter 2. Media were concentrated 10 fold using a Millipore Ultrafree centrifugal filter device with a semi-permeable membrane with a

molecular weight exclusion of 10 kDa. Protein in aliquots of concentrated media proportional to the amount of cellular protein were separated by SDS-PAGE (10%) and transferred to nitrocellulose membranes. The amounts of TGH and albumin were assessed by immunoblotting using respective polyclonal antibodies as in Section 4.2.4.

6.2.3 Production and analysis of a non-glycosylated secreted TGH mutant

The lone glycosylation site in TGH was disrupted by site directed mutagenesis using the QuikChange Site-Directed Mutagenesis Kit according to the manufacturer's instructions. The template for mutagenesis was ΔR-TGH in the pCI-neo plasmid. Primer sequences were forward: 5'-g c ttt gtg aag CAA gcc acc tcg tac c-3' reverse: 5'-g gta cga ggt ggc TTG ctt cac aaa gc-3'. The mutated codon in the primers is shown in upper case. The result from PCR was a construct coding for a non-glycosylated, secreted mutant (N79QΔR-TGH).

The N79QΔR-TGH construct was stably transfected into McArdle RH7777 cells using Lipofectamine2000 in the procedure described in Section 2.6. Cells from a resulting cell line and untransfected cells were plated on 60 mm dishes and incubated overnight. Media were changed to 2 mL of serum free DMEM and incubated for an additional 12 h. Media were cleared of cell debris by centrifugation and lipase activity in 100 μL was measured. To determine if activity was due to TGH in the media, GR148672X (a TGH specific inhibitor) was included in concurrent assays at a final concentration of 10 μM. Cells were scraped into PBS and briefly sonicated. Fifty μg of cellular protein and 65 μL of media were subjected to denaturing SDS-PAGE (10%). Immunoblots were performed to detect TGH protein as laid out in Chapter 2.

The N79QΔR-TGH construct was also transiently transfected into Cos-7 cells. Plasmid DNA was introduced to Cos-7 cells using Lipofectamine2000 as described was above for stable transfection of McArdle RH7777 cells. Then 28 h after transfection, media were changed to 2 mL of serum free DMEM and incubated for an additional 12 h. Cells and media were collected as above for analysis of lipase activity and the presence of TGH by immunoblot.

6.2.4 Specific enzyme activity of TGH and TGH mutants

In order to determine if mutations to TGH engineered during this investigation affected enzymatic activity, *in vitro* lipase assays were performed using quantities of cell lysates with normalized amounts of TGH. McArdle RH7777 cells expressing *wt*-TGH, ΔR-TGH or N79QΔR-TGH were analyzed as per Section 4.2.11.

6.3 Results

6.3.1 Glycosylation is not required for enzymatic activity

TGH is a glycoprotein (Alam *et al.*, 2002b) with a single *N*-glycosylation site at asparagine 79. To investigate the requirement of glycosylation for catalytic activity of TGH, ΔR-TGH expressing cells were treated with tunicamycin, a potent inhibitor of *N*-glycosylation. Immunoblots performed on media samples from ΔR-TGH expressing cells treated with tunicamycin showed 2 bands for ΔR-TGH, representing the glycosylated and non-glycosylated enzyme (Figure 6-1). Without tunicamycin, only fully glycosylated TGH is secreted (Figure 6-1). The amount of albumin in the media did not change with tunicamycin treatment, indicating the production of proteins and their movement through the secretory pathway was not appreciably altered. Despite the substantial reduction in glycosylation of ΔR-TGH with tunicamycin treatment (>60%), there was no decrease in TGH activity in the media compared to untreated ΔR-TGH expressing cells (Figure 6-2). This data demonstrates that glycosylation is not required for enzymatic activity of TGH, and also suggests correct folding of the protein during synthesis is maintained.

To further define a potential requirement of glycosylation for TGH, a secreted mutant in which the glycosylation site had been disrupted was created (N79QΔR-TGH). The mutation of asparagine to glutamine is conservative, maintaining the charge on this amino acid residue while preventing glycosylation. The construct was used for stable transfection of McArdle RH7777 cells. The immunoblot in Figure 6-3 A shows the N79QΔR-TGH protein was synthesized

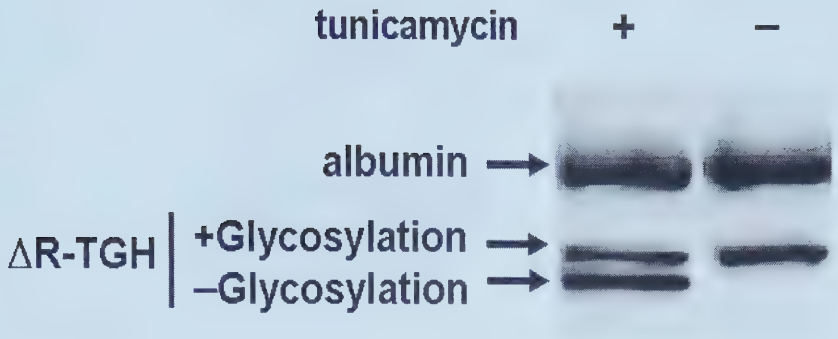


Figure 6-1 Tunicamycin decreases glycosylation of ΔR-TGH. McArdle RH7777 cells stably expressing ΔR-TGH were treated with tunicamycin as described in Materials and Methods. Relative amounts of both ΔR-TGH and albumin were assessed in 10 fold concentrated media by immunoblotting using respective polyclonal antibodies.

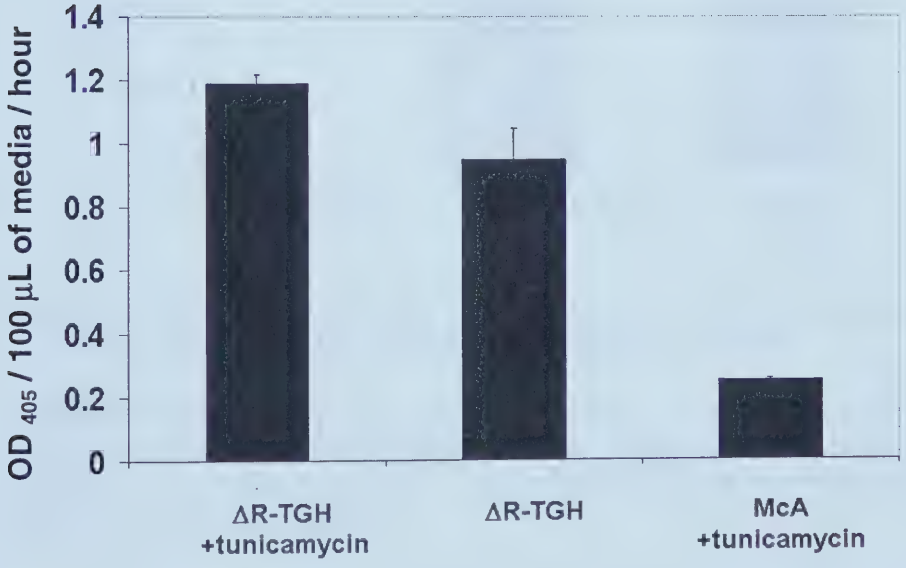


Figure 6-2 Tunicamycin does not reduce ΔR-TGH activity. Lipase activity in 10x concentrated media of tunicamycin treated McArdle RH7777 cells (McA) and McArdle RH7777 cells expressing ΔR-TGH were assessed using p-nitrophenyl laurate as substrate. Data are the mean ± SD of triplicate samples and are representative of three independent experiments.

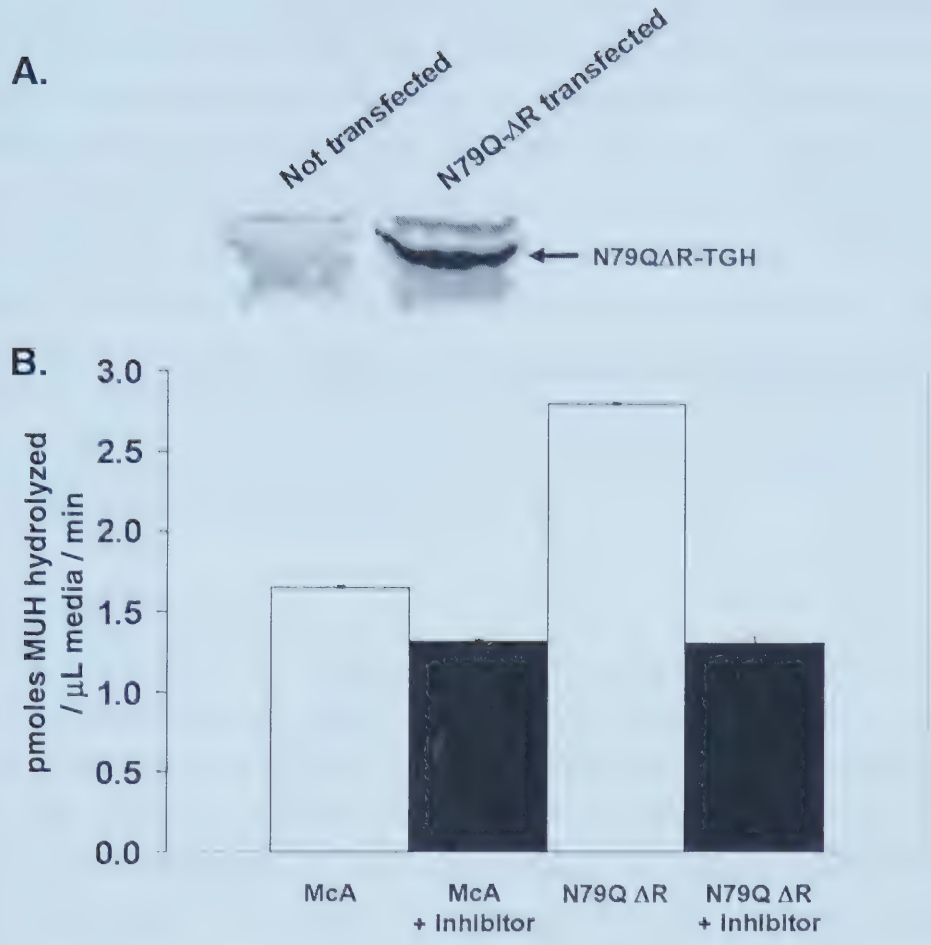


Figure 6-3 A non-glycosylated, secreted mutant of TGH is produced in an active state from transfected McArdle RH7777 cells.

A. McArdle RH7777 cells were stably transfected to produce a secreted glycosylation mutant of TGH (N79QΔR-TGH). N79QΔR-TGH protein was detected in the media after an overnight incubation via immunoblot using anti-TGH polyclonal antibodies.

B. Lipase activity in media of N79QΔR-TGH stably transfected McArdle RH7777 cells were determined using 4-methylumbelliferyl heptanoate (MUH) as substrate. A TGH specific inhibitor was included in the assay to resolve whether increases in lipase activity were legitimately due to the production of TGH. Data characterize activity found in media via liberation of 4-methylumbelliferone. Shown are the mean ± SD of triplicate samples and are representative of three independent experiments.

and secreted. The transfected cells exhibited ~2 fold increase in lipase activity in media over samples from untransfected cells (Figure 6-3 B). To show that the additional activity was due to the presence of N79QΔR-TGH in the media, a TGH specific inhibitor was included in the assay (GR148672X from Chapter 3). The inhibitor reduced the relative lipase activity to the same levels observed with media from untransfected cells. Similar results were derived from Cos-7 cells transiently transfected with the N79QΔR-TGH construct (Figure 6-4). Assays using normalized amounts of TGH show that specific activity is not substantially altered by removal of the C-terminal retention sequence or mutation of the glycosylation site (Figure 6-5). These studies confirm that glycosylation of TGH is not a required modification for catalytic activity.

6.4 Discussion

TGH was shown to be glycosylated with a single *N*-glycosylation site on asparagine 79 (Alam *et al.*, 2002b). *N*-linked glycosylation is required for proper folding of many proteins as it can facilitate the interaction with chaperones that bind to the carbohydrate moiety, assisting folding and isomerization especially with respect to disulfide bond formation (reviewed in (Ellgaard *et al.*, 1999)). Glycosylation has also been shown to be required for maximum *in vitro* catalytic activity for other carboxylesterases (Kroetz *et al.*, 1993). A rabbit carboxylesterase very similar to TGH has been crystallized recently in a fully glycosylated form and in the presence of a known substrate molecule (Bencharit *et al.*, 2002). This enzyme shares the *N*-glycosylation site at asparagine 79 with TGH, and has an additional *N*-glycosylation site at asparagine 389. The crystal structure indicates that two *N*-acetyl glucosamine groups are found on asparagine 79; this pattern is likely conserved in TGH. The structure also suggests that asparagine 389 is involved in the catalytic event by providing a flexible region from which products of catalysis can exit the "pocket" that contains the active site. Because TGH does not share a homologous glycosylation site at asparagine 389, exit of the products of hydrolysis might be dissimilar between these two enzymes.

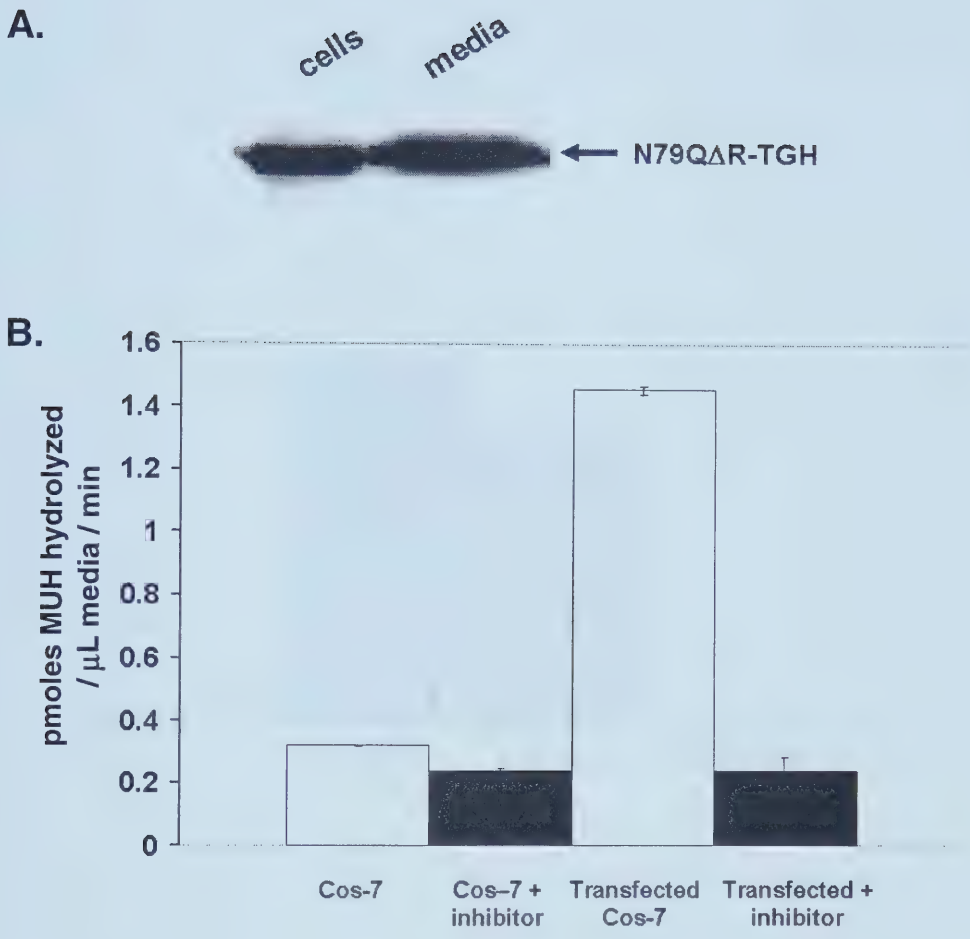


Figure 6-4 A non-glycosylated, secreted mutant of TGH is produced in an active state from transfected Cos-7 cells.

A. Cos-7 cells were transiently transfected to produce a secreted glycosylation mutant of TGH (N79QΔR-TGH). N79QΔR-TGH protein was detected in the cells and media via immunoblot. Untransfected Cos-7 cells do not express TGH.
B. Lipase activity in media of N79QΔR-TGH transiently transfected Cos-7 cells was determined using 4-methylumbelliferyl heptanoate (MUH) as substrate. Data were produced as per Figure 6-3, where McArdle RH7777 cells were used.

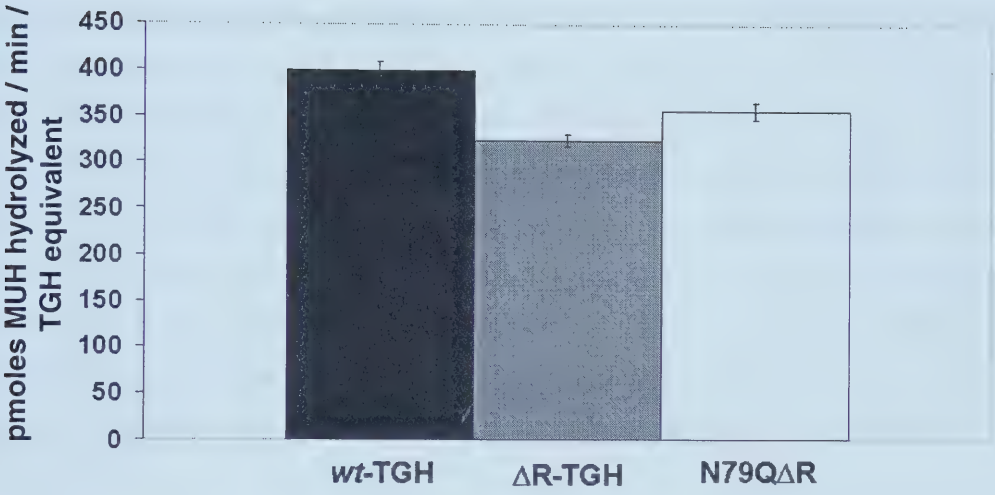


Figure 6-5 Specific activity of TGH and mutants. The quantity of *wt*-TGH or mutants of TGH in cell lysates from stably transfected McArdle RH7777 cells were normalized via immunoblots using fluorescent secondary antibodies. Lysates containing equal amounts of TGH were used in an *in vitro* lipase activity assay with 4-methylumbelliferyl heptanoate (MUH) as substrate. Data are the mean ± SD of 3 samples and are representative of three independent experiments.

Recently, the crystal structure of the first human carboxylesterase was published (Bencharit *et al.*, 2003b). The enzyme is known as egasyn, and the resulting structural data indicates the glycosylation pattern at asparagine 79 also consists of two *N*-acetyl glucosamines.

Treatment of hepatoma cells expressing a secreted but active mutant of TGH (ΔR-TGH described in Chapter 4) with tunicamycin did not alter the activity of the secreted protein or the secretion of albumin, a non-glycosylated secreted protein. Mutation of the glycosylation site confirmed that TGH does not require this modification for catalytic activity. Lipase activity assays with normalized amounts of *wt*-TGH, ΔR-TGH or N79QΔR-TGH indicate that lack of the C-terminal four amino acids and/or glycosylation does not affect specific enzyme activity. Further, this data suggests that both of these TGH mutants are folded properly, even in a non-glycosylated state (Figure 6-5). Therefore, glycosylation appears to be a dispensable modification to TGH. This observation was somewhat of a surprise, as glycosylation often has beneficial consequences towards efficient production and folding of proteins manufactured in the ER.

Chapter 6:

Post-translational modifications of TGH

B) Phosphorylation

The majority of the information presented in this Chapter is included in a submitted manuscript

Gilham, D., K. R. Perreault, C. F. B. Holmes, D. N. Brindley, D. E. Vance, and R. Lehner (2005) Insulin, glucagon and fatty acid treatment of hepatocytes does not result in phosphorylation or changes in activity of triacylglycerol hydrolase *Biochim. Biophys. Acta*

6.5 Introduction

It is recognized that the majority of VLDL associated TG is derived from intra-hepatic stores rather than from *de novo* synthesized lipids (see Section 1.3.4). Although the requirement for stored TG to undergo lipolysis and re-esterification in order to be secreted in association with VLDL has been well established, an understanding of the molecular events in this process remain obscure. TGH is involved in mobilizing hepatic stored TG for secretion on VLDL; modulating the ability of TGH to mobilize stored lipids represents a potentially regulated and rate limiting step in VLDL assembly.

Phosphorylation of a number of other lipases and carboxylesterases has been demonstrated, and this modification affects their subcellular localization, access to substrate or enzymatic activity (Anthonsen *et al.*, 1998; Egan *et al.*, 1992; Ghosh and Grogan, 1989; Ghosh and Grogan, 1990; Ghosh *et al.*, 1990; Natarajan *et al.*, 1997; Pasqualini *et al.*, 2000; Verine *et al.*, 2001; Zimmermann *et al.*, 2004). To date, little is known about regulation of TGH activity. The phosphorylation of rat TGH on Ser 506 has been described (Wallace *et al.*, 2001), however, this serine residue is not conserved in the human, monkey or mouse orthologs (Figure 1-7). Hence the increase of rat TGH activity by phosphorylation cannot be directly translated to these other species.

In this Chapter, it is hypothesized that human TGH undergoes phosphorylation in response to hormones including insulin and glucagon or by increasing intracellular TG content with excess fatty acids. VLDL secretion from liver has been shown to respond to fed state and plasma insulin levels (Bjornsson *et al.*, 1992; Chirieac *et al.*, 2000; Salhanick *et al.*, 1991; Sparks and Sparks, 1994). Glucagon can be considered an opposing hormone to insulin. Potentially, TGH activity could be sensitive to the amount of TG storage, or to extracellular signals like insulin and glucagon, and the consequent modulation of this activity may regulate the provision of TG for assembly with primordial apoB containing particles. Several consensus sequences for phosphorylation by a variety of kinases are present in the human TGH primary amino acid sequence. Phosphorylation of human TGH was probed using radio-labeled inorganic

phosphate in intact cells after stimulation with insulin or glucagon or augmenting TG storage pools with oleic acid. Further, *in vitro* lipase activity of TGH was examined after treating cells with these agents and after treating isolated TGH with alkaline phosphatase. Despite the precedents of other lipases undergoing phosphorylation events with functional consequences, phosphorylation of TGH as well as alterations of *in vitro* activity were not detected under all conditions examined.

6.6 Materials and Methods

6.6.1 . Reagents

Phosphate free DMEM, and alkaline phosphatase were purchased from Invitrogen Canada, Burlington, ON, Canada). Sodium orthovanidate, sodium fluoride, tetrasodium pyrophosphate (phosphatase inhibitors) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) and monoclonal anti-flag antibody covalently attached to agarose beads were from Sigma-Aldrich, Oakville, ON, Canada. The monoclonal M2 anti-flag antibody used in immunoblots was from Stratagene, La Jolla, CA. Polyclonal antibodies for mitogen-activated protein (MAP) kinase (also known as extracellular signal-regulated kinase (ERK)) and a monoclonal anti-phosphotyrosine antibody were obtained from Santa Cruz Biotechnology, Santa Cruz, CA. A monoclonal antibody for phospho-MAP kinase was obtained from Cell Signaling Technology, Beverly, MA. Anti-rabbit secondary antibodies conjugated to the fluorescent dye IR800 were from Rockland Immunochemicals, Gilbertsville, PA, and anti-mouse secondary antibodies conjugated to Alexa Fluor 680 were from Molecular Probes, Eugene, OR. [^{33}P] orthophosphate was purchased from ICN Biomedicals, Irvine, CA, while [^{32}P] orthophosphate was from Amersham Biosciences, Baie d'Urfé, QC, Canada.

6.6.2 Insulin induced stimulation of MAP kinase phosphorylation

McArdle RH7777 cells on 60 mm plates and grown to approximately 70% confluence, washed with PBS and incubated in serum free DMEM for 15 min.

Media were aspirated, then cells were incubated in either DMEM only (control), DMEM supplemented with 100 nM insulin, or DMEM supplemented with 10 nM glucagon. Incubation was continued for 30 min, then cells were scraped into ice-cold buffer containing 20 mM Tris pH 8, 1% CHAPS and phosphatase inhibitors (1 mM sodium orthovanidate, 50 mM sodium fluoride and 5 mM tetrasodium pyrophosphate). Some dishes that received insulin treatment were harvested in a similar buffer lacking the phosphatase inhibitors. Cells were disrupted by brief sonication and centrifuged at 2,700 x*g* at 4°C for 5 min to pellet unlysed cells and insoluble cell debris. Supernatants were transferred to clean tubes and protein concentrations determined using Bio-Rad protein assay reagent. Cell lysates containing 60 μg of protein were adjusted to consistent volumes with respective buffers. Aliquots from cells treated with insulin and harvested with or without the phosphatase inhibitors were treated with 20 U of alkaline phosphatase and incubated at 37°C for 15 min. Denaturing electrophoresis sample buffer was added to all samples, which were then boiled for 5 min and subjected to SDS-PAGE (10%). Proteins were transferred to a nitrocellulose membrane. Immunoblots for MAP kinase and phospho-MAP kinase were performed using corresponding antibodies in accordance to the supplier's protocols. Fluorphor-labeled secondary antibodies were employed to allow quantitation of fluorescence intensity from the immunoblots using a Li-Cor Odyssey Infrared Imaging System. This experimental design was developed with Dr. David Brindley.

6.6.3 Protein phosphatase-1 activity

McArdle RH7777 cells were plated on 60 mm plates and grown to 70% confluence. Cells were washed with PBS and incubated in DMEM ± 10 nM glucagon for 30 min. Cells were harvested in Tris buffered saline (TBS; 20 mM Tris pH 7.4, 137 mM NaCl), sonicated briefly, then insoluble cell debris and unlysed cells were sedimented by centrifugation. Protein concentrations in the homogenates were determined. Protein phosphatase-1 activity in the cell lysates was measured via the dephosphorylation of [^{32}P] labeled phosphorylase *a* using

an assay described previously (Cohen *et al.*, 1988; Holmes, 1991; MacKintosh, 1993). Briefly, 10 μL aliquots of buffer containing 50 mM Tris pH 7.5, 0.1 mM EDTA, and 0.2% 2-mercaptoethanol were combined with 10 μL of cell lysates and incubated at 30°C for 10 min. [^{32}P] labeled phosphorylase *a* (10^6 cpm/nmol), in a buffer consisting of 50 mM Tris pH 7.5, 0.1 mM EDTA, 15 mM caffeine and 0.2% 2-mercaptoethanol was added as a substrate. The reaction was terminated after 10 min with the addition of 100 μL of 20% trichloroacetic acid (w/v). Samples were placed on ice for at least 2 min, and then centrifuged at 12,000 xg for 2 min to sediment the precipitated proteins. Because the substrate is a protein that is radio-labeled near the amino-terminus (serine-14), active proteases may have been provided in the cell lysates that could generate short radio-labeled peptides that do not precipitate with trichloroacetic acid. In order to exclude the participation of intracellular proteases generating short [^{32}P] labeled peptides, versus bona fide protein phosphatase-1 activity releasing labeled inorganic phosphate, acid-molybdate extraction of inorganic phosphate was performed (MacKintosh, 1993). 200 μL of acid phosphate (1.25 mM KH_2PO_4 in 0.5 M H_2SO_4) were added, and an organic extraction was carried out via the addition of 300 μL of heptane/2-butanol (1:1 v/v). Then 100 μL of 5% (w/v) ammonium molybdate was added and the solution mixed by vortexing. Upon separation, the radioactivity associated with phosphomolybdic acid in 300 μL of the organic phase was determined by scintillation counting.

6.6.4 Production of flag-epitope tagged TGH

The flag-epitope consists of the octapeptide DYKDDDDK. A sequence coding for this epitope tag was introduced to the human *wt*-TGH cDNA by PCR as described in Section 4.2.9. The sequence coding for the flag-epitope was incorporated immediately upstream of the region coding for the amino acid sequence –HIEL, which remained at the extreme C-terminus of the corresponding protein. The PCR product included the coding region of the cDNA only; untranslated regions were eliminated. The PCR product was ligated into the mammalian expression plasmid pCI-neo after digestion of both plasmid and

PCR product with appropriate restriction endonucleases. The entire insert was sequenced to ensure errors were not introduced during PCR and the segment coding for the epitope tag had been appended correctly. This construct, as well as the empty plasmid, were used for stable transfection of McArdle RH7777 cells using the procedure outlined in Section 2.6. All experiments described in this Chapter were performed with cell lines demonstrating robust expression of flag-epitope tagged TGH protein by immunoblot.

6.6.5 Flag-epitope tagged TGH is catalytically active

Cells stably transfected with flag-epitope tagged TGH or with empty pCI-neo plasmid were grown to approximately 70% confluence, scraped into PBS and lysed by sonication. Protein concentrations in the lysates were determined. The presence of TGH in aliquots of the cell lysates containing 50 μg of protein was probed by immunoblot using anti-flag M2 monoclonal antibody according to the manufacturer's protocol. Lipase activity was determined in an assay including 10 μg of protein from cell lysates and MUH as a substrate (see Section 2.9).

6.6.6 Immunoblots for phosphotyrosine

In three 60 mm dishes, McArdle RH7777 cells stably transfected with flag-epitope tagged TGH were grown to approximately 70% confluence. Cells were washed with PBS and incubated in serum free DMEM for 15 min. Media were aspirated, then cells were incubated in either DMEM only (control), DMEM supplemented with 100 nM insulin, or DMEM supplemented with 10 nM glucagon. Incubation was continued for 30 min, then cells were scraped into ice-cold buffer containing 20 mM Tris pH 8, 1% CHAPS and the phosphatase inhibitors listed above. Cells were disrupted by brief sonication and unlysed cells and insoluble cell debris were sedimented by centrifugation. Supernatants were transferred to clean tubes and their protein concentrations determined. Immunoprecipitation (IP) of TGH was performed by combining 750 μg of protein from each lysate with 35 μL of agarose beads conjugated to anti-flag antibody.

Tubes were incubated at 4°C on a rotating rack for 16 h.IP beads were sedimented by centrifugation at 1,000 x*g* for 2 min. The supernatants were recovered, while IP beads were washed with the same buffer the cells were scraped and lysed into and then twice with TBS. Beads were resuspended in denaturing electrophoresis sample buffer and boiled for 5 min. Proteins that had been associated with the beads were separated by SDS-PAGE (10%). An aliquot of an IP supernatant was included on the gel to serve as a control for immunoblots. Proteins were transferred to nitrocellulose membranes, and then visualized by staining them with Ponceau S. The membrane was cut in order to perform immunoblots using anti-phosphotyrosine or anti-flag antibodies concurrently on equivalent samples. The anti-phosphotyrosine antibody is described by the manufacturer to provide specific detection of phosphotyrosine containing proteins and does not cross-react with phosphoserine or phosphothreonine.

6.6.7 Phosphate labeling of proteins in transfected McArdle RH7777 cells

McArdle RH7777 cells stably expressing flag-epitope tagged TGH or stably transfected with the empty pCI-neo plasmid were plated in 35 mm dishes at a density of $3x10^5$ cells/dish and grown overnight. One plate each of pCI-neo or flag-epitope tagged TGH transfected cells were incubated with DMEM containing 0.4 mM oleate complexed with 0.5% fatty acid free BSA for 4 h to augment TG storage (see Section 2.7), and thus markedly increase the substrate pools conceivably available to TGH. All of the dishes were rinsed once with phosphate free DMEM and then incubated in subsequent aliquots of this media for 30 min. Media were removed, phosphate free DMEM containing 40 μCi of [^{33}P] orthophosphate per milliliter were added and incubation was continued for 40 min. Insulin or glucagon was added to a final concentration of 100 nM or 10 nM respectively to a set of stable transfectants. Incubation was continued for another 30 min, culminating in a total of 70 min incubation with radio-labeled phosphate. Cells were washed three times with TBS and scraped into 500 μL of IP buffer (defined in Section 3.2.9) containing a protease inhibitor cocktail (Roche Diagnostics) and the phosphatase inhibitor combination used above. Cells were

disrupted by sonication and insoluble cell debris and unlysed cells were sedimented by centrifugation. The supernatants were transferred to new tubes and 100 μL of TBS were added, resulting in detergent concentrations of 0.58% Triton X-100 and 0.08% SDS. Each tube received 35 μL of agarose beads conjugated to anti-flag antibody in order to immuno-isolate flag-epitope tagged TGH as in the procedure conducted above. The IP supernatants were recovered, and the beads were washed twice with IP buffer then once with TBS. Beads were resuspended in denaturing electrophoresis buffer and placed in an 80°C water bath for 45 sec. Proteins in the cell homogenates, immunoprecipitates and in aliquots from the supernatants were separated by SDS-PAGE, followed by transfer to nitrocellulose membranes. Proteins on the membranes were visualized by staining with Ponceau S. Immunoblots for TGH were performed using polyclonal antibodies generated in our laboratory (Alam *et al.*, 2002a) and with anti-flag antibody. Radioactivity on the membranes resulting from incorporation of labeled phosphate into proteins was detected by exposure to phosphorimaging screens for 4 days, then reading the screens using a Bio-Rad Molecular Imager FX phosphorimager and Bio-Rad Quantity One software.

The phosphate labeling procedures and TGH IPs were repeated using a longer incubation period and 67 μCi of [^{32}P] orthophosphate per milliliter of media, rather than 40 μCi of [^{33}P] orthophosphate, as the [^{32}P] isotope has a stronger and more easily detected radioactive emission. Incubations with the radio-labeled phosphate were extended from 40 min to 5.5 h. Hormones were introduced as above, and cells incubated with them for an additional 30 min, culminating in a total of 6 h incubation with radio-labeled phosphate. The extended incubation period should allow the detection of a basal phosphorylation state of TGH as well as a hormone stimulated phosphorylation state. Analysis was performed via IP, SDS-PAGE, protein staining, immunoblot and phosphorimaging as above.

6.6.8 Effect of alkaline phosphatase on TGH enzyme activity

McArdle RH7777 cells stably transfected with empty plasmid or with flag-epitope tagged TGH were plated in 60 mm dishes and grown overnight. Three dishes of each cell line were incubated for 4 h in serum free DMEM containing 0.4 mM oleate and 0.5% BSA. The remaining dishes were washed once with PBS, then for 30 min in serum free DMEM. Three of these plates from each respective cell line were then treated with serum free DMEM ± 100 nm insulin or 10 nM glucagon and all dishes were then incubated for another 30 min. Cells in the various treatment groups were washed once with TBS, and scraped into buffer containing 20 mM Tris pH 8, 1% CHAPS and the phosphatase inhibitor combination denoted above. One dish of each stable cell line that was treated with insulin was scraped into a similar buffer lacking the phosphatase inhibitors. Cells were disrupted by sonication, then centrifuged to pellet insoluble cell debris and unlysed cells. Supernatants were transferred to new tubes and protein concentrations determined. Aliquots containing 450 μg of protein from each treatment group containing phosphatase inhibitors were isolated for IP of TGH using anti-flag antibody conjugated to agarose beads as in the procedure described above. The agarose beads were then washed once with the same buffer used when scraping the cells from the dishes, then twice with 20 mM Tris pH 8 in order to remove detergent and phosphatase inhibitors. Following washes, 50 μL of 20 mM Tris pH 8 were added. The samples were divided into 2 aliquots of 35 μL; one aliquot was treated with 8 U of alkaline phosphatase for 15 min at 37°C. Lipase activity assays were performed on 4 μL of the beads after these treatments using MUH as a substrate.

6.7 Results

6.7.1 Phosphorylation of MAP kinase in response to insulin

First, verification that McArdle RH7777 cells respond to insulin and that signaling pathways for this hormone are intact in this cell line was determined. Insulin is known to activate MAP kinase via a signal transduction cascade that results in phosphorylation of MAP kinase (Bevan, 2001; Combettes-Souverain

and Issad, 1998). The phosphorylation state of this protein was investigated via immunoblots using antibodies that recognize MAP kinase protein, as well as an antibody specific for phosphorylated MAP kinase. As shown in Figure 6-6 A, the antibodies used recognize two isoforms of MAP kinase. Overlaying the images generated using these two antibodies (merge) results in overlap of the bands specific for MAP kinase and phospho-MAP kinase. Quantitation of fluorescence intensity provided by fluorophores conjugated to the secondary antibodies indicates consistent amounts of MAP kinase were loaded on the gel (Figure 6-6 B). The ratio of the fluorescence intensity from the phospho-MAP kinase blot to the intensity from the total MAP kinase immunoblot is shown in Figure 6-6 C. The phospho-MAP kinase blot shows markedly more of the phosphorylated species in insulin treated cells (Figure 6-6 A Lanes 2 and 4; Figure 6-6 C). MAP kinase in McArdle RH7777 cells was phosphorylated in response to insulin but not glucagon. This response to insulin shows that McArdle RH7777 cells are sensitive to this hormone. An insulin signaling cascade was also shown to be intact in McArdle RH7777 cells by another group using a similar immunoblotting technique for protein kinase B (also know as Akt1) (Au *et al.*, 2004). The intensity of the band for phospho-MAP kinase was reduced to levels seen in unstimulated cells upon treatment of cell lysates with alkaline phosphatase without phosphatase inhibitors (Figure 6-6 A Lane 5; Figure 6-6 C), demonstrating that the phosphatase used in these experiments was capable of removing phosphate groups from proteins. Further, alkaline phosphatase was not able to dephosphorylate MAP kinase in the presence of the phosphatase inhibitors (Figure 6-6 A Lane 4; Figure 6-6 C), indicating these were effective agents in protecting against endogenous as well as exogenous phosphatases during the manipulations performed.

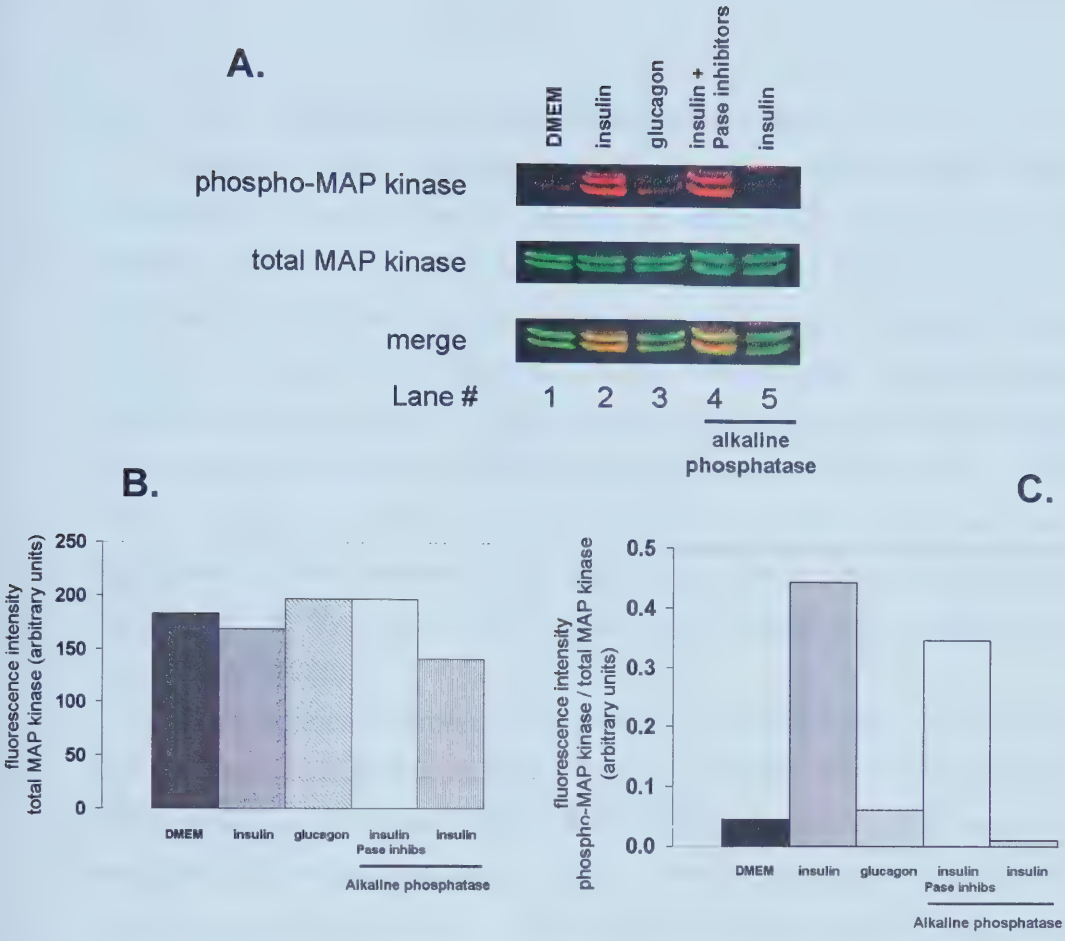


Figure 6-6 An insulin signaling cascade involving mitogen-activated protein (MAP) kinase is intact in McArdle RH7777 cells.
McArdle RH7777 cells were stimulated with insulin or glucagon, then homogenized in buffer ± a combination of phosphatase (Pase) inhibitors described in Materials and Methods. Some samples containing 60 μg of protein from cell lysates ± phosphatase inhibitors were incubated with alkaline phosphatase. Proteins in all samples were then separated by SDS-PAGE, transferred to nitrocellulose membranes and probed by immunoblot using primary antibodies directed against MAP kinase and phosphorylated (phospho)-MAP kinase concurrently and secondary antibodies conjugated to fluorophors.
A. Immunoblots using antibodies that recognize phospho-MAP kinase, total MAP kinase and a merge of the two images.
B. Quantitation of total MAP-kinase.
C. The ratio of fluorescence intensity from the phospho-MAP kinase to the total MAP kinase immunoblots.
Concept and antibodies for this figure were provided by Dr. David Brindley.

6.7.2 Protein phosphatase-1 activity in response to glucagon

Assays to verify McArdle RH7777 cells respond to glucagon were also performed. Glucagon has a number of effects on glycogen metabolizing enzymes in the liver (Bollen *et al.*, 1998). The net result is that glucagon promotes the mobilization of glucose from stored glycogen. Glucagon binds to a cell surface receptor and induces a signal transduction cascade, resulting in elevated levels of intracellular cyclic adenosine monophosphate and activation of cyclic adenosine monophosphate-dependent protein kinase (PKA). Activated PKA can phosphorylate protein phosphatase inhibitor-1 (as well as other substrates). When phosphorylated, this inhibitor binds to protein phosphatase-1 (also known as glycogen phosphorylase phosphatase) and reduces its activity (Cohen *et al.*, 1988).

The presence of protein phosphatase inhibitor-1 mRNA and protein in rat liver and isolated hepatocytes has been demonstrated (Aleem *et al.*, 2001), and the phosphorylation / dephosphorylation of this inhibitor in rat liver has also been described (Shenolikar and Steiner, 1984). After treatment of McArdle RH7777 cells with or without glucagon, activity of protein phosphatase-1 was determined using a [^{32}P] labeled substrate specific for this enzyme (i.e. glycogen phosphorylase *a*). The liberation of [^{32}P] was measured after an incubation of the labeled substrate with cell lysates. As shown in Figure 6-7, a 20% decrease ($P<0.03$) in protein phosphatase-1 activity was measured in lysates from glucagon treated cells, indicating that glucagon specific cell signaling events are intact in McArdle RH7777 cells. The magnitude of this decrease in enzyme activity is consistent with what other investigators have reported in response to glucagon in hepatic systems (Farkas *et al.*, 1986; Stalmans *et al.*, 1990; Toth *et al.*, 1988). Therefore, McArdle RH7777 cells appear to be a suitable model system for studying signaling events initiated by insulin and glucagon.

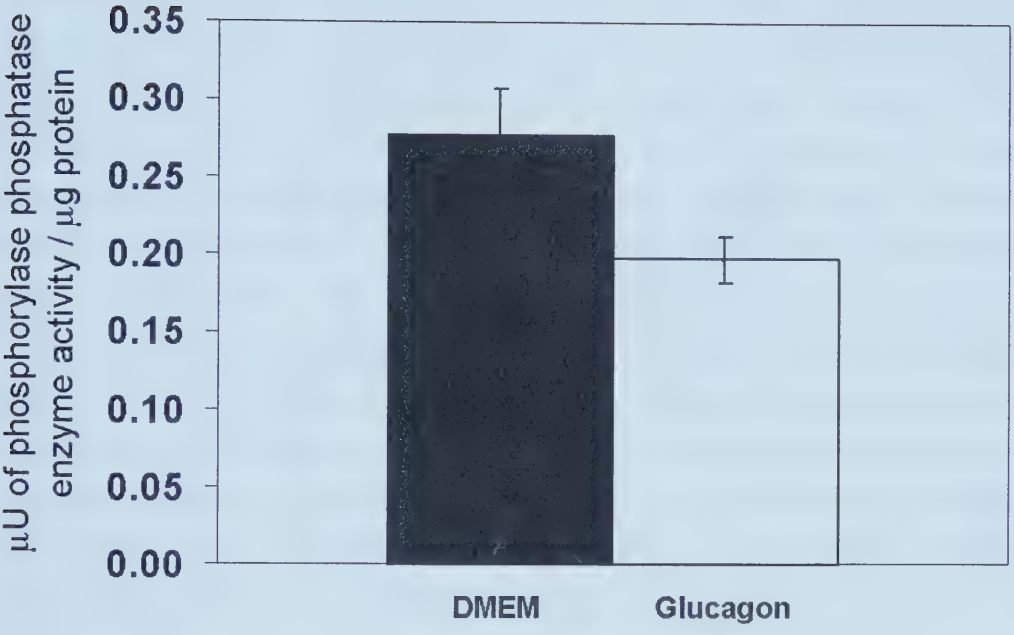


Figure 6-7 A glucagon induced signal transduction cascade involving protein phosphatase-1 is intact in McArdle RH7777 cells. Protein phosphatase-1 activity was measured in McArdle RH7777 cell lysates after treating cells with 10 nM glucagon or media alone (DMEM) in an enzyme assay described in Materials and Methods. One microUnit (μU) of protein phosphatase-1 activity releases 1 pmole of phosphate from phosphorylase *a* per min. Data are the mean ± standard deviation of triplicate samples. $P<0.03$
This figure was developed in collaboration with Kathleen Perreault and Dr. Charles Holmes.

6.7.3 Epitope tagging of recombinant TGH

Three-dimensional modeling of TGH (Alam *et al.*, 2002b) based on the X-ray crystallography coordinates of acetylcholine esterase (Sussman *et al.*, 1991) indicate the C-terminal tail of TGH is exposed to the surface of the protein and relatively unstructured. This model is supported by the more recently published crystal structures of a carboxylesterase from rabbit that is similar to TGH (Bencharit *et al.*, 2002), as well as the first crystal structure of a human carboxylesterase (Bencharit *et al.*, 2003b). The C-terminal region was thus utilized in our experiments to engineer a flag-epitope tag into the TGH protein in order to facilitate IPs. The position of the flag-epitope tag was specifically designed such that the endogenous amino acid sequence –HIEL was maintained at the extreme C-terminus of the protein. This tetrapeptide has been shown to be a functional ER retrieval sequence for TGH (described in Chapter 4), and it must be positioned at the extreme C-terminus to operate in this capacity (Robbi and Beaufay, 1991). McArdle RH7777 cells do not endogenously synthesize TGH (Lehner and Vance, 1999). Immunoblots using an anti-flag antibody demonstrates the presence of flag-epitope tagged TGH in stably transfected McArdle RH7777 cell lysates (Figure 6-8 A). Lipase activity assays show that flag-epitope tagged TGH is catalytically active (Figure 6-8 B). These data demonstrate that inclusion of the epitope did not affect the ability of this enzyme to catalyze the hydrolysis of substrates. Endogenous activity towards MUH in McArdle RH7777 cells is quite low (Figure 5-8 B), and our results indicate that transfected TGH becomes the most active neutral lipase present in these cells.

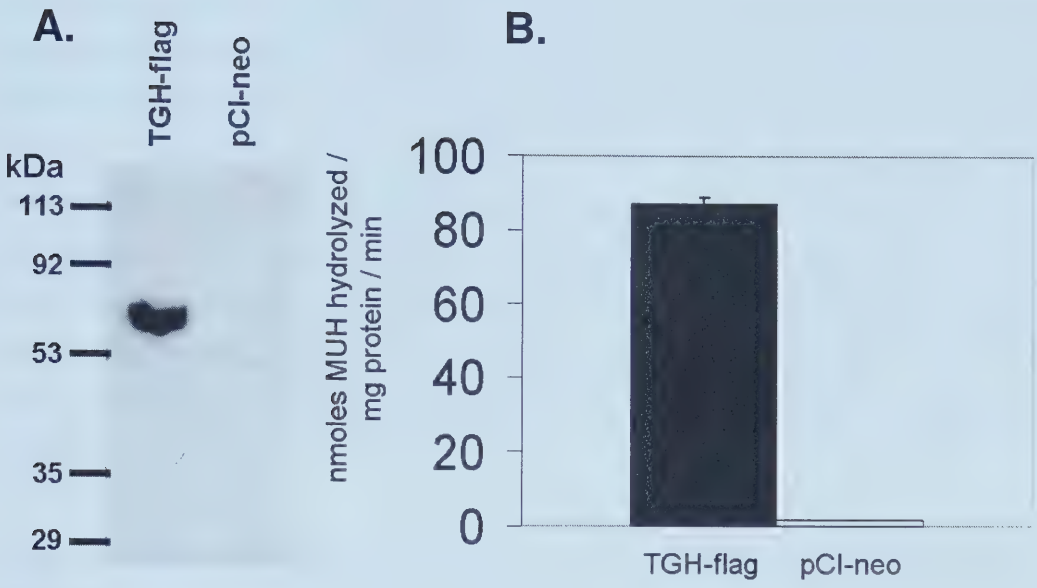


Figure 6-8 Expression of flag-epitope tagged TGH in McArdle RH7777 cells.

A. McArdle RH7777 cells were stably transfected with flag-epitope tagged TGH (TGH-flag) or empty pCI-neo plasmid. The presence of TGH protein in cell homogenates was determined by immunoblot using monoclonal M2 anti-flag antibody.

B. Activity in cell homogenates was determined using the fluorogenic substrate 4-methylumbelliferyl heptanoate (MUH). Data are the mean ± standard deviation of triplicate samples derived from three independent dishes of cells.

6.7.4 Probing TGH for phosphotyrosine via immunoblot

Analysis of the TGH primary amino acid sequence revealed the presence of several serine / threonine phosphorylation consensus motifs and one potential tyrosine phosphorylation site (tyrosine 118; Table 6-1). To investigate a possible tyrosine phosphorylation event on TGH, McArdle RH7777 cells were treated with insulin or glucagon, IPs for flag-epitope tagged TGH were conducted, then immunoblots using anti-phosphotyrosine and anti-flag antibodies were performed. The anti-phosphotyrosine antibodies did not recognize TGH as bearing a phosphotyrosine residue, although the IP supernatants showed signals for other tyrosine phosphorylated proteins. Based on this data, one must conclude that insulin or glucagon treatment does not result in phosphorylation of tyrosine 118 in TGH.

6.7.5 Radio-labeling of phosphorylated proteins in McArdle RH7777 cells and immunoprecipitation of flag-epitope tagged TGH

The next studies explored whether insulin, glucagon or increased cellular TG stores would lead to changes in overall TGH phosphorylation state. Detection of phosphorylated proteins was accomplished by radio-labeling with [^{33}P] orthophosphate. A large number of proteins in the cell homogenates were phosphorylated during these procedures, both in basal conditions (no stimulus) and after receiving the indicated treatments, as observed by phosphorimaging (Figure 6-9 A). TGH was immunoprecipitated from these homogenates with antibodies specific for a flag-epitope tag engineered into the protein, then analyzed by SDS-PAGE, transferred to nitrocellulose membranes, stained for protein, immunoblotted and subjected to phosphorimaging. The presence of TGH on the nitrocellulose membranes was readily observed in IPs from TGH-flag transfected cells using Ponceau S protein stain (Figure 6-9 B Lanes 1-4 top panel), and its identity was confirmed by immunoblot using anti-TGH (Figure 6-9 B middle panel). To determine if the immunoprecipitated TGH was phosphorylated, phosphorimaging was performed (Figure 6-9 B bottom panel).

phosphorylation event	TGH amino acid sequence with consensus for phosphorylation	amino acid position
Tyrosine Phosphorylation:	KLSEDCL**Y**	118
Serine/Threonine Phosphorylation:	**T**PAD	123
	STYD	150
	STGD	179
(casein kinase II motifs)	**T**EEE	290
	TVID	321
	TPEE	331
	SEEE	486

Table 6-1 Consensus sequences for phosphorylation in human TGH. The amino acid residue that could receive the phosphate group is shown within the consensus sequence in bold and underlined text (top). Its position in the TGH primary amino acid sequence is shown as the amino acid number with the initiating methionine designated as 1. The potential tyrosine phosphorylation site is expanded (bottom) to indicate its proximity to the lid domain. Phosphorylation of this residue could conceivably regulate opening of the lid, access of substrates to the active site and thus regulate enzyme activity.

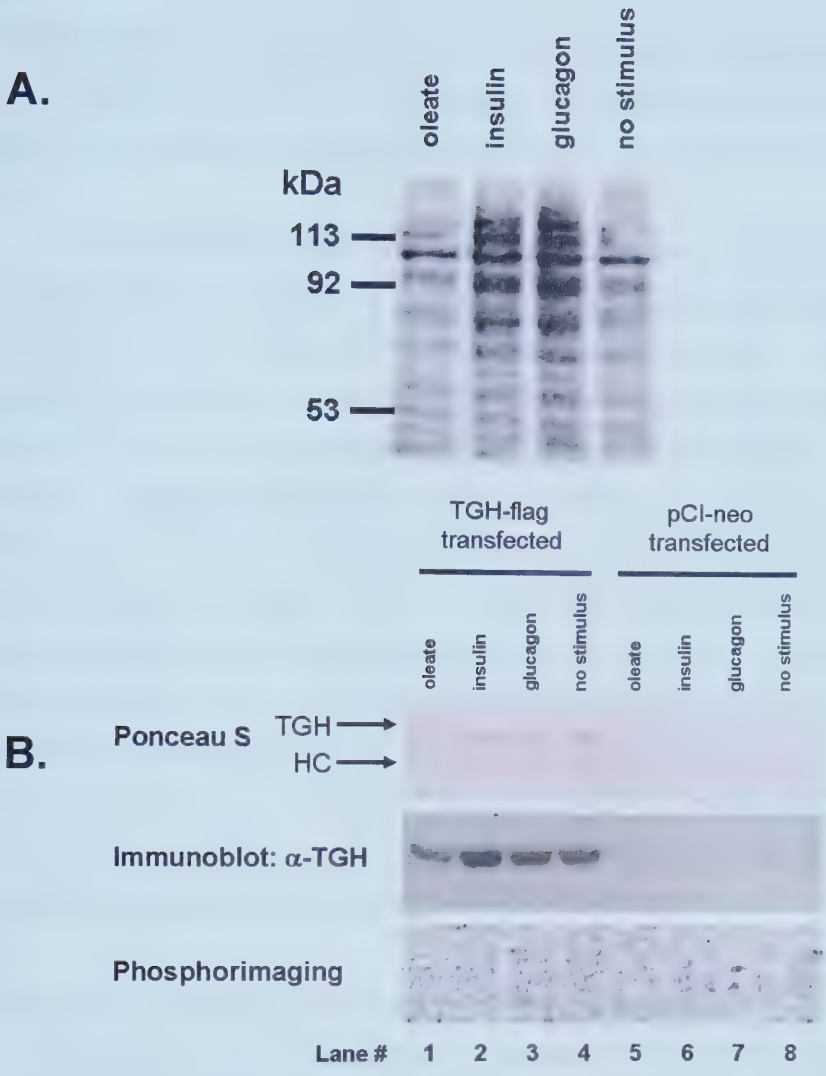


Figure 6-9 Immunoprecipitation and phosphorimaging of TGH from [^{33}P] orthophosphate labeled cells. McArdle RH7777 cells were incubated with 40 μCi/mL media (60 μCi/dish) $H_3[^{33}PO_4]$ along with 0.4 mM fatty acid (oleate), insulin, glucagon or media alone (no stimulus). TGH was immunoprecipitated from cell homogenates and analyzed by the indicated methods.

A. Proteins in cell homogenates were separated by SDS-PAGE, transferred to nitrocellulose and analyzed by phosphorimaging.

B. TGH was immunoprecipitated from cells receiving the various treatments above, subjected to SDS-PAGE and transferred to nitrocellulose membranes. Membranes were stained with Ponceau S (top), probed by immunoblot using anti-TGH polyclonal antibodies (middle) and for radio-labeled proteins by phosphorimaging (bottom). The band for TGH and the immunoglobulin heavy chain (HC) are indicated.

Phosphorylation of TGH could not be detected after labeling cells with [^{33}P] orthophosphate over a 70 min incubation period, although the radio-label was found in association with many other proteins in the cell homogenates (Figure 6-9 A).

Phosphorylation of proteins is a dynamic process, as the phosphate group can be added and removed. It is possible that the phosphorylation of TGH was not detected due to short and inadequate labeling periods. To eliminate this possibility, experiments were repeated with a substantially longer incubation period (6 h) with radio-labeled orthophosphate as described in Materials and Methods. The longer incubation period may allow the detection of a basal, as well as a hormone stimulated, phosphorylation state for TGH. These experiments also employed [^{32}P], rather than [^{33}P], in order to take advantage of a stronger, and therefore more easily detected radioactive emission. As in the experiments with [^{33}P] orthophosphate, numerous phosphorylated proteins were detected in cell homogenates. Immunoprecipitated TGH on the nitrocellulose membrane was easily recognized with Ponceau S protein stain (Figure 6-10, Lanes 1-4 top panel), but phosphorylation of immuno-isolated TGH was not detected by phosphorimaging (Figure 6-10 bottom panel).

6.7.6 Alkaline phosphatase treatment of immunoprecipitated TGH

In vitro lipase assays were conducted with TGH that had been immunoprecipitated from cells that had received oleic acid (4 h incubation), insulin or glucagon (30 min) or no treatment. After IP, the beads were divided into two aliquots: one received alkaline phosphatase while the other did not. Subsequent *in vitro* lipase activity assays demonstrate that TGH is catalytically active on IP beads under the experimental conditions employed. As shown in Figure 6-11, TGH immuno-purified from transfected cells exhibited similar specific enzyme activity regardless of hormone or fatty acid treatments, and incubations with alkaline phosphatase did not alter this activity.

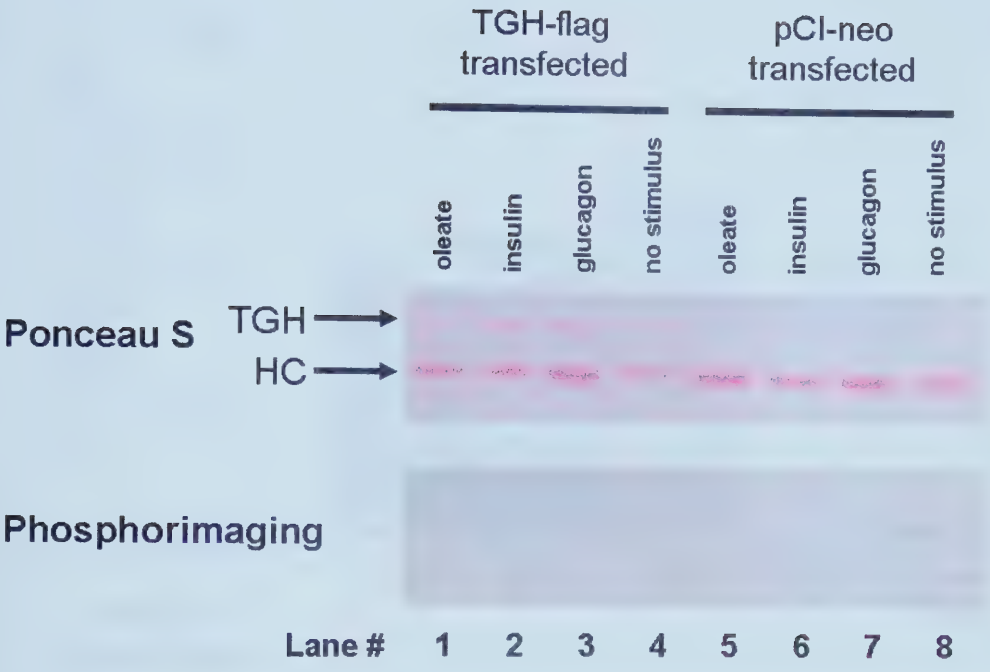


Figure 6-10 Immunoprecipitation and phosphorimaging of TGH from [^{32}P] orthophosphate labeled cells. The orthophosphate radio-labeling of cellular proteins, as well as the immunoprecipitation and phosphorimaging of TGH was repeated using 67 μCi/mL media (100 μCi/dish) [^{32}P] orthophosphate and an extended labeling period (6 h). TGH was immunoprecipitated from cells receiving the various treatments indicated, subjected to SDS-PAGE and transferred to nitrocellulose membranes. Membranes were stained with Ponceau S (top) and for radio-labeled proteins by phosphorimaging (bottom). The band for TGH and the immunoglobulin heavy chain (HC) are indicated.

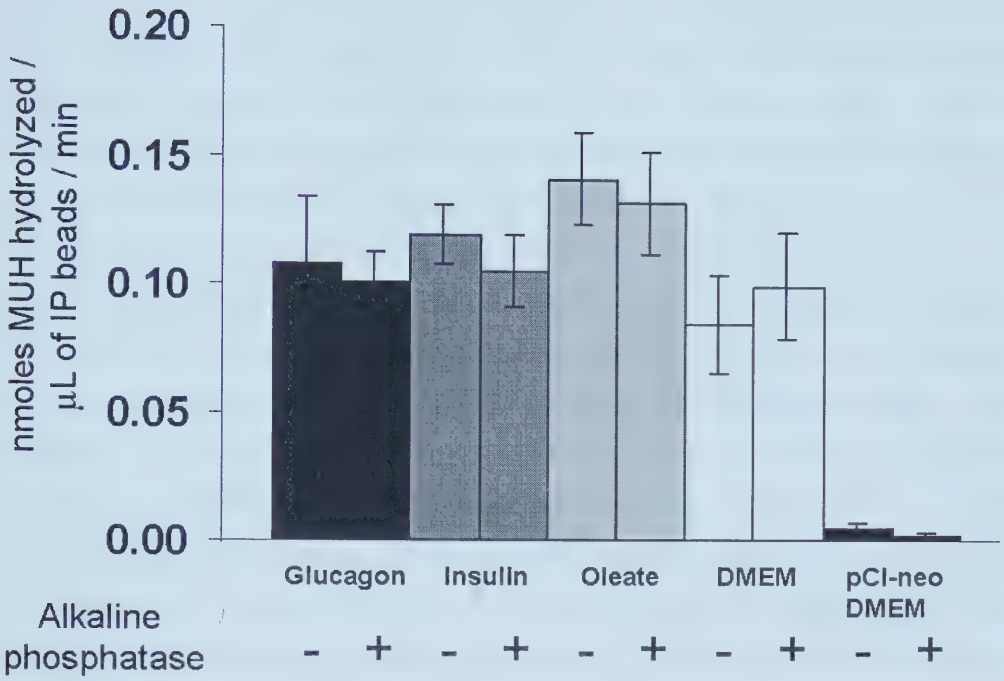


Figure 6-11 Effect of insulin, glucagon and oleic acid on TGH *in vitro* lipase activity. Stably transfected McArdle RH7777 cells were incubated with fatty acids, insulin, glucagon or media alone (DMEM) as indicated in Materials and Methods, then TGH was immunoprecipitated under non-denaturing conditions from cell lysates. The immunoprecipitation beads from each treatment group were divided into 2 equal aliquots. One aliquot from each group received alkaline phosphatase for 15 min. The *in vitro* lipase activity was assayed on all samples using 4-methylumbelliferyl heptanoate (MUH) as substrate. Data are the mean ± standard deviation of triplicate samples.

6.8 Discussion

It has been well established that insulin is a key regulator of apoB secretion in various models (Dashti and Wolfbauer, 1987; Pullinger *et al.*, 1989; Salhanick *et al.*, 1991; Sparks *et al.*, 1986). Plasma insulin levels are now recognized as having a major physiological impact on the regulation of plasma TG concentrations, particularly in the post-prandial state (reviewed in (Gibbons *et al.*, 2002; Sparks and Sparks, 1994)). Impairment of insulin action on the liver results in failure to suppress VLDL production, and contributes to the high levels of VLDL commonly observed in insulin resistance or type 2 diabetes (Gibbons, 1986). Data from HepG2 cells demonstrated that apoB levels are not regulated by insulin at the transcriptional level (Dashti *et al.*, 1989; Pullinger *et al.*, 1989), but rather insulin inhibits hepatic VLDL secretion through a mechanism involving intracellular degradation of *de novo* synthesized apoB (Chirieac *et al.*, 2002). The insulin induced decrease in hepatic VLDL secretion also does not appear to be related to MTP expression as insulin had no effect on hepatic MTP mRNA levels in rats (Ameen and Oscarsson, 2003). TGH is involved in mobilizing stored lipid for secretion on VLDL. As shown in Chapter 3, inhibition of TGH leads to decreased secretion of apoB accompanied by increased apoB degradation. Therefore, experiments were designed to determine if insulin's effect on hepatic apoB secretion may be in part mediated via regulation of TGH enzyme activity and ability to mobilize neutral lipids from storage pools for lipidation of primordial apoB containing particles.

Currently little is known about regulation of TGH activity. Similarly, mechanisms regulating the mobilization and channeling of neutral lipids from hepatic storage depots have not been adequately defined. Previously at least six distinct bands for purified TGH were observed in isoelectric focusing studies, all in the acidic region (p*I* 5.3-5.8), and it was hypothesized that these bands represented either different glycosylation or phosphorylation states (Lehner and Verger, 1997).

Many lipases and esterases have their active site buried under secondary structure elements that must change conformation to allow a substrate to access the active site. These secondary structure elements have been called caps, lids or flaps (Holmquist, 2000) and have important roles in regulating accessibility of substrates into the catalytic crevice. A search for potential phosphorylation sites in the TGH primary structure: (http://www.embl-heidelberg.de/predictprotein/predictprotein.html) revealed a tyrosine phosphorylation site at a critical position (Tyr 118; Table 6-1), as it lies adjacent to the hinge domain of the lid (amino acids 87-116, Figure 1-7, 1-9). Considering this location, one may hypothesize that phosphorylation of this tyrosine residue could regulate opening of the lid and interaction of TGH with substrates. Further analysis of the primary structure of TGH revealed seven additional consensus sequences for phosphorylation by casein kinase II (Table 6-1.). A population of casein kinase II (a serine / threonine kinase) has been shown to exist in the lumen of the ER (Edelman *et al.*, 1987; Sfeir and Veis, 1995; Singh and Huang, 1985) and produce functional modifications to proteins in this compartment, including a lipase (Cala and Jones, 1994; Pasqualini *et al.*, 2000; Verine *et al.*, 2001). Presently factors and stimuli involved in activation of this kinase are ill-defined (Meggio and Pinna, 2003). Insulin has been reported to activate casein kinase II (Kim *et al.*, 1998), and this hormone's impact on lipid metabolism and VLDL secretion have been well documented. Glucagon is recognized as opposing the effects of insulin, and therefore these hormones were prime candidates for modulating the participation of TGH in VLDL assembly, potentially though a casein kinase II mediated mechanism.

Regulation of lipase function by phosphorylation is best documented for hormone sensitive lipase (HSL). Recall from Chapter 1 that HSL is a cytosolic lipase and has been shown to be principle in mobilizing TG in adipose (reviewed in (Holm *et al.*, 2000; Langin *et al.*, 1996; Londos *et al.*, 1999a)). PKA mediated phosphorylation of HSL induces a translocation from a dispersion in the cytosol to the surface of intracellular lipid droplets (Brasaemle *et al.*, 2000a; Clifford *et al.*, 2000; Egan *et al.*, 1992). A novel lipase in adipose tissue has recently and

independently been described by several groups. The enzyme has been termed adipose triglyceride lipase (ATGL; (Zimmermann *et al.*, 2004)), as well as phospholipase A2-ζ (Jenkins *et al.*, 2004), desnutrin (Villena *et al.*, 2004) and previously in the database as transport-secretion protein 2.2 (TTS-2.2)). ATGL is also phosphorylated, but in contrast to HSL, this is not mediated by PKA nor does it induce translocation of ATGL to a lipid droplet surface (Zimmermann *et al.*, 2004).

In addition to HSL and ATGL, phosphorylation of bile salt-dependant lipase (BSDL; also known as pancreatic cholesterol esterase) has been demonstrated. BSDL is a cholesterol esterase found in pancreatic secretions in the duodenal region of the intestine (Hui, 1996; Wang and Hartsuck, 1993). This enzyme participates in the hydrolysis of dietary cholesterol esters and lipid-soluble vitamins (Howles *et al.*, 1996; Lombardo and Guy, 1980). During its synthesis, BSDL undergoes *N*-glycosylation (Sugo *et al.*, 1993) and the lipase then associates with the lumenal leaflet of the ER membrane. Dissociation from membranes and secretion into the intestine is accomplished by phosphorylation by casein kinase II (Pasqualini *et al.*, 2000; Verine *et al.*, 2001). Phosphorylation of BSDL is believed to depend on a protein kinase cascade, however, as indicated above, mechanisms for regulation of casein kinase II activity have not yet been determined (reviewed in (Meggio and Pinna, 2003)). Similarly, it has been shown that the oncofetal variant of BSDL is also dependant on phosphorylation for exit from the ER and secretion (Verine *et al.*, 2001). A mutant of this enzyme that could not be phosphorylated failed to exit from intracellular compartments, but remained catalytically active towards BSDL substrates.

The phosphorylation of Ser 506 in rat liver carboxylesterase, identical to rat TGH, has been reported (Wallace *et al.*, 2001). Phosphorylation is mediated by PKA and protein kinase C, which stimulates a 2-2.5 fold increase in cholesteryl ester hydrolase activity. The stimulation can be reversed by dephosphorylation catalyzed by a Mg^{2+} dependent protein phosphatase. Introducing the PKA phosphorylation site into rat lung carboxylesterase by site-

directed mutagenesis conferred inducible cholesteryl ester hydrolase activity to this enzyme (Wallace *et al.*, 2001). However, Ser 506 is not present in mouse, rabbit, monkey or human TGH (Figure 1-7), and therefore the implication of rat TGH phosphorylation cannot be universally applied. The current study utilized human TGH, and thus the ability / inability of this protein to be modified by phosphorylation had not been previously described.

The concept that lipolytic activity in the liver may not be regulated by insulin has been suggested (Brown and Gibbons, 2001; Gibbons *et al.*, 2004; Wiggins and Gibbons, 1992), and it was concluded that recruitment of intracellular TG for VLDL assembly is suppressed rather than lipolysis or mobilization from storage pools. However, the above studies were performed in cells treated with dexamethasone, which results in dramatic decrease of TGH expression and activity (Dolinsky *et al.*, 2004a), and therefore the regulation of TGH activity by insulin remained to be determined. The mechanism of dexamethasone induced reduction of TGH expression in mice was determined to be reduced mRNA stability (Dolinsky *et al.*, 2004a). This effect was mediated through the 3' untranslated region of the mRNA. The construct used in our study of human TGH lacked the untranslated regions. Further, protein expression from the pCI-neo plasmid was driven by the cytomegalovirus promoter, which does not contain glucocorticoid response elements. Hence unlike mouse liver, TGH protein expression should not be affected by glucocorticoids. Our data show that insulin, glucagon or supply of fatty acids does not lead to TGH phosphorylation nor changes in the enzyme's activity and support the conclusions of Wiggins and Gibbons (Wiggins and Gibbons, 1992) that hepatic TG turnover is not regulated by insulin or glucagon signaling.

Chapter 6:

Post-translational modifications of TGH

C) Acylation

6.9 Introduction

Regulation of TGH activity or interactions with its substrate pool remains unclear. Another mechanism of transiently potentiating TGH affinity for hydrophobic surfaces like membranes or lipid droplets is via reversible acylation of a cysteine residue. The amino acid sequence of human TGH contains five cysteine residues. Our three-dimensional model predicts two intramolecular disulfide bridges leaving Cys 390 available for covalent acylation. However, neitherchemical modification of Cys 390 nor mutation to alanine affected lipolytic activity of TGH, suggesting no functional participation of Cys 390 in substrate binding or hydrolysis.

It has been recently reported that a porcine carboxylesterase homologous to TGH can undergo cysteine acylation (Smialowski-Fleter *et al.*, 2002). The deduced amino acid sequence of TGH has five cysteine residues. Our three-dimensional model structure suggests that two disulfide bridges are formed between C87-C116 and C274-C285 (Alam *et al.*, 2002b). The remaining cysteine residue at position 390 would be available for modification and appears to be surface oriented. Since acylation of cysteine residues is reversible, an attractive hypothesis is that adsorption of TGH to the endoplasmic reticulum associated lipid droplets may be mediated by this post-translational modification. Alternately it is available for intermolecular disulfide bonds resulting in homo or heterodimers. In this Chapter, the possible roles of the putative NLBD and Cys 390 residue in TGH in catalysis are explored using site directed mutagenesis and biochemical approaches.

6.10 Materials and Methods

6.10.1 Reagents

Iodoacetamide and anti-flag M2 antibody covalently attached to agarose beads were from Sigma-Aldrich, Oakville, ON, Canada. SF-900 II SFM media was from Invitrogen, Burlington, ON, Canada. Nickel-nitrilotriacetic acid (Ni-NTA) resin for purification of recombinant polyhistidine-tagged proteins and the anti-His4 monoclonal antibody were from Qiagen, Valencia, CA.

The oligonucleotides used to generate mutant TGH constructs were

C390A forward: cc tat ccc ctt gtt **GCC** att gct aag gaa ctg att cc

C390A reverse: gg aat cag ttc ctt agc aat **GGC** aac aag ggg ata gg

The mutated codon in both primers is indicated in bold capital letters.

6.10.2 Recombinant plasmids

This study was initiated with a full length TGH cDNA construct with a C-terminal polyhistidine tag in the pFASTBAC plasmid (Invitrogen) described previously (Alam *et al.*, 2002a). In previous studies, the polyhistidine tagged protein remained intracellular in Sf-9 insect cells (Alam *et al.*, 2002b), despite the prediction that it would be secreted and the data from mammalian cells presented in Chapter 4 with ΔR-TGH. This plasmid was the template for mutagenesis to generate a C390A mutant using the primer pair indicated above. Specifically, the template for mutagenesis included the coding region of the human TGH cDNA that lacked the region for C-terminal sequence HIEL, but included an additional sequence for a hexa-histidine tag to facilitate purification. Recombinant baculovirus was generated from these plasmids as described in the BAC-TO-BAC baculovirus system (Invitrogen) and used for expression of mutant and *wt*-TGH in Sf-9 cells as described below.

The insert encoding the C390A mutation was subcloned in the pCI-neo mammalian expression vector (Promega Corporation, Madison, WI.) and was used for expression in Cos-7 cells as described below.

6.10.3 Expression of recombinant TGH and its mutants in Sf-9 cells

Sf-9 cells were cultured at 28°C with GIBCO BRL Sf-900 II supplemented with 5% FBS, 50 U/mL penicillin and 50 μg/mL streptomycin. For expression of *wt*-TGH and TGH with the point mutation C390A, $1x10^6$ Sf-9 cells were seeded in 100 mm tissue culture dishes. Cells were infected with recombinant baculovirus at a multiplicity of infection of 1 to 5, incubated for 72 to 96 h after infection, harvested in PBS and analyzed for protein content with Bio-Rad protein assay reagent. Expression of TGH in the cell lysates was assessed by immunoblots

using polyclonal anti-TGH antibodies. The resulting nitrocellulose membranes were also stained with Ponceau S. Lipase activity in Sf-9 cell lysates was measured spectrophotometrically in an assay using *p*-nitrophenyl laurate as substrate (Section 2.9). Membrane and cytosolic fractions were prepared by centrifugation as described in Section 3.2.2.

6.10.4 In vitro fatty acylation of TGH

McArdle RH7777 cells stably expressing flag-epitope tagged TGH, introduced in Chapter 6 B were utilized in these studies to probe for acylation. Because this TGH protein can be immuno-isolated, determination of an acylation event on the isolated species can be accomplished. Investigation of microsomal protein acylation was performed essentially as described (Lehner and Kuksis, 1993a) with some modifications. In brief, microsomes from McArdle RH7777 cells stably expressing flag-epitope tagged TGH were isolated by centrifugation. 125 μg of microsomal protein was incubated with 0.5 μCi of [^{14}C] oleoyl-CoA (Amersham Lifesciences, Baie d'Urfé, PQ, Canada) in 50 mM Tris (pH 7.6) in a final volume of 100 μL at 37°C for 30 min. Membranes were solubilized with 50 mM Tris (pH 7.6), 150 mM NaCl, 1% TX-100, 1 mM EDTA containing Complete Protease Inhibitor Cocktail (Roche) and the solubilized proteins were resolved from insoluble debris by centrifugation. Although palmitate is the fatty acid most commonly found on thiol-acylated proteins, thiol-acylation is not specific for palmitate, and other fatty acid moieties can be found including thio-oleoylation (Schroeder *et al.*, 1996). Flag-epitope tagged TGH was immunoprecipitated from supernatants with 30 μL of anti-flagM2 antibody attached to agarose beads overnight at 4°C on a rotating rack. The immunoprecipitates were washed 3 times with 50 mM Tris (pH 7.6), 150 mM NaCl, 1% TX-100, 1 mM EDTA. Proteins remaining in the IP supernatants were precipitated with trichloroacetic acid (Peterson, 1977). Proteins in trichloroacetic acid and immuno- precipitates were treated with non-reducing electrophoresis buffer (without β-mercaptoethanol), separated by SDS-PAGE and transferred to a nitrocellulose membrane. Gels also contained [^{14}C] labeled methylated protein standards

(Amersham Lifesciences). The membranes were probed for the presence of TGH with anti-TGH polyclonal antibodies. After immunoblotting, the membranes were exposed to a Bio-Rad phosphorimaging screen and the radioactivity covalently associated with proteins was analyzed using a Bio-Rad Molecular Imager FX Phosphorimager. Finally, proteins on the membrane were stained with Ponceau S, the areas corresponding to TGH and the [^{14}C] labeled protein standards were excised and the associated radioactivity was determined by scintillation counting.

6.10.5 Chemical modification of the free cysteine residue in TGH

Chemical modification of the free cysteine residue present in TGH was accomplished by treating purified TGH for 45 min with iodoacetamide at the concentrations indicated in the figure legend on ice prior to assessment of the lipolytic activity.

6.11 Results

6.11.1 Putative role of free cysteine 390 in TGH

The TGH primary amino acid sequence contains five cysteine residues (Cys 87, Cys 116, Cys 274, Cys 285 and Cys 390). Two disulfide bridges are predicted to form between Cys 87-116 and Cys 274-285, leaving Cys 390 free based on the proposed three dimensional structure of TGH (Figure 1-9). Cys 390 could be available for the formation of intermolecular disulfide bonds. However, treatment of purified TGH with or without reducing agent in the electrophoresis buffer did not affect its electrophoretic mobility in SDS-PAGE (Figure 6-12 A), indicating that no covalent intermolecular association via a disulfide bond exists between TGH and itself or other proteins that would give rise to either homo- or hetero- dimers. In addition, alkylation of Cys 390 with iodoacetamide did not result in alteration of TGH lipolytic activity suggesting that Cys 390 is not involved in catalysis (Figure 6-12 B).

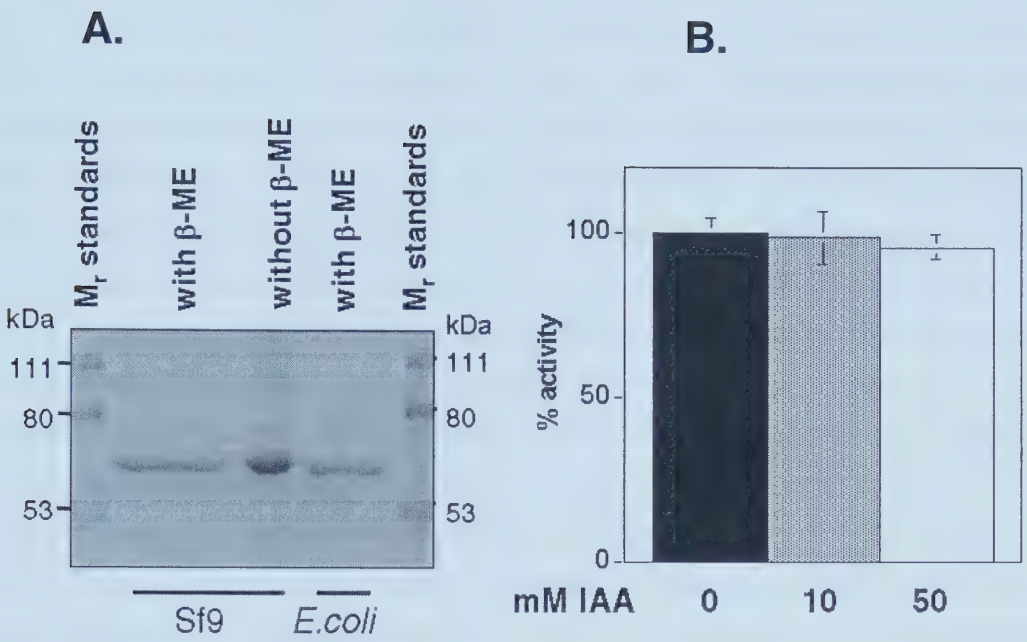


Figure 6-12 Effect of β-mercaptoethanol and iodoacetamide on electrophoretic mobility and activity of *wt*-TGH. Recombinant human TGH was purified from baculovirus infected Sf9 cells under native conditions and subjected to various treatments.

A. Purified *wt*-TGH expressed in Sf9 cells and in E. coli were treated with SDS-PAGE sample buffer with and without β-mercaptoethanol (β-ME), subjected to SDS-PAGE followed by staining with Coomassie brilliant blue R-250. M_r standards, pre-stained molecular mass standards

B. *wt*-TGH purified from Sf9 cells was treated with various concentrations of iodoacetamide on ice for 45 minutes prior to determination of the lipolytic activity. Relative activities using *p*-nitrophenyl laurate are shown as a percentage of untreated *wt*-TGH (100%).
Figure 6-12 was provided by Dr. Mustafa Alam.

It is well known that protein acylation (myristoylation and palmitoylation) influences a wide spectrum of structural and functional features of proteins. These modifications can alter localization, association with membranes (Resh, 1999) or enzyme activity (Berthiaume *et al.*, 1994). Cysteine-acylation is a reversible post-translational modification occurring through the thiol group of this amino acid residue (Milligan *et al.*, 1995; Mumby, 1997). It has been reported that a porcine carboxylesterase with high homology to TGH undergoes thio-acylation (Smialowski-Fleter *et al.*, 2002). Since TGH contains an available cysteine residue, investigation into whether the enzyme undergoes this post-translational modification was performed. As shown in Figure 6-13 A, incubations of microsomes isolated from McArdle RH7777 cells stably expressing flag-eptiope tagged TGH with [^{14}C] oleoyl-CoA resulted in acylation of proteins present in the supernatant after immunoprecipitation of TGH, however, the acylation of immunoprecipitated TGH was not detected. This observation was confirmed by scintillation counting of the regions of the nitrocellulose membrane containing the TGH band (Figure 6-13 B). The results demonstrate that Cys 390 of TGH is not playing a crucial role in catalysis or association with membranes or other substrate pools via acylation. To further support these observations, a C390A TGH mutant was constructed and expressed in Sf9 cells using a baculovirus protein expression system. Lipase activity determinations were used to assess the catalytic functionality of the mutant enzyme. Figure 6-14 A and 6B show that the C390A mutant TGH protein was expressed in baculovirus infected Sf9 cells. Activity measurements with *p*-nitrophenyl laurate employing the same amounts of *wt*-TGH indicated no differences in the ability of C390A TGH to hydrolysis of this lipid ester (Figure 6-14 C). These data support the conclusion that Cys 390 is not essential for TGH activity, and does not promote membrane interaction or covalent oligomerization.

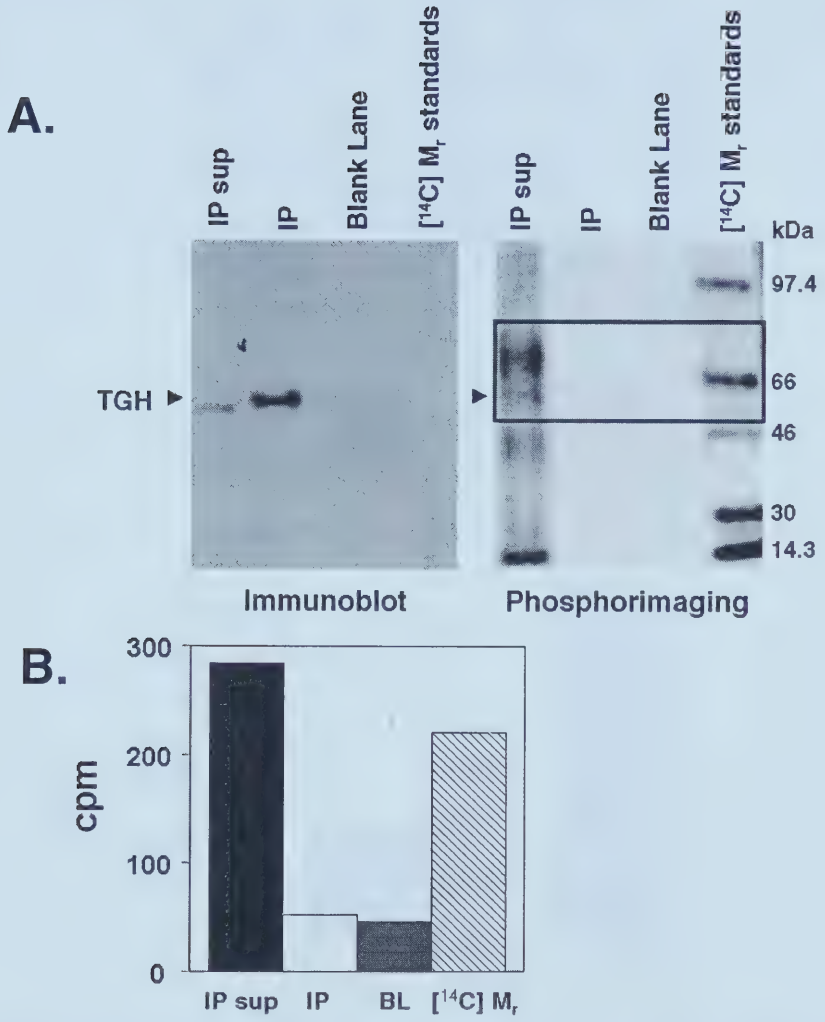


Figure 6-13 TGH is not acylated in McArdle RH7777 cells. Microsomal proteins from McArdle RH7777 cells stably expressing flag-epitope tagged TGH were incubated with [^{14}C] oleoyl-CoA as described in Materials and Methods, followed by immunoprecipitation of TGH with monoclonal anti-flag antibody. Both immunoprecipitated (IP) and remaining non-immunoprecipitated microsomal proteins (IP sup) were subjected to non-reducing SDS-PAGE followed by transfer to a nitrocellulose membrane.
A. Immunoblot and phosphorimaging;
B. Radioactivity in the area marked by a box in (**A.**) associated with immunoprecipitated TGH (IP), non-immmunoprecipitated proteins (IP sup), blank lane (BL) and [^{14}C] M_r standards after excision of the areas of the nitrocellulose membrane, followed by scintillation counting.
This figure was developed in collaboration with Dr. Mustafa Alam.

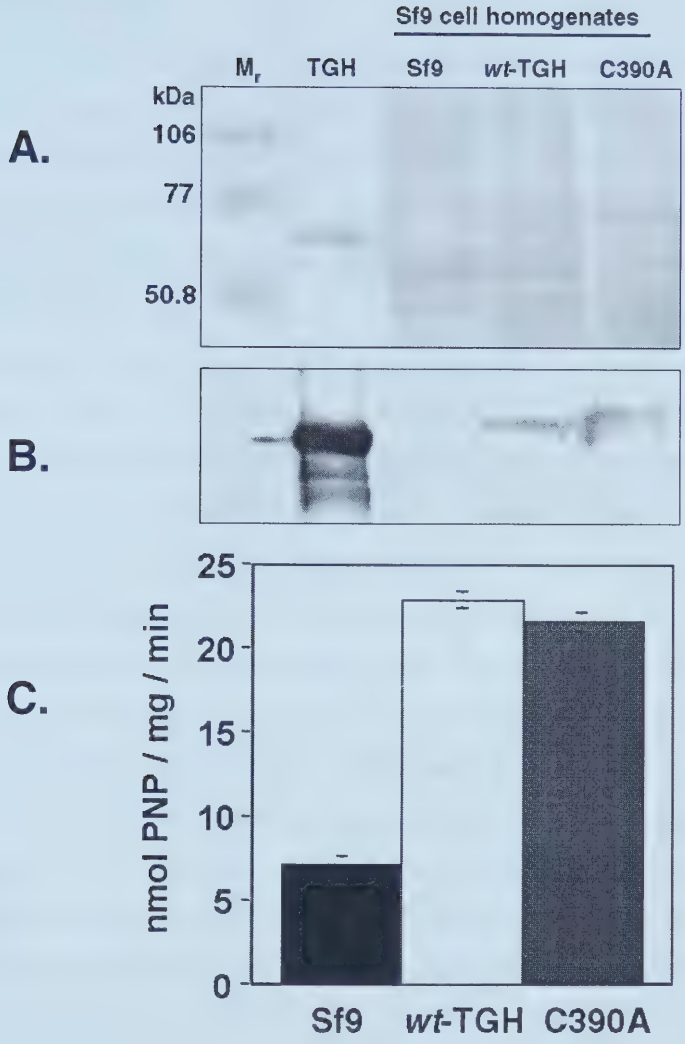


Figure 6-14 Expression and activity of the C390A TGH mutant. Homogenates were prepared from Sf9 cells or Sf9 cells expressing *wt*-TGH or the C390A mutant by recombinant baculovirus infection as described in Materials and Methods. Proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane.

A. Ponceau S staining: M_r; pre-stained molecular weight standards TGH; purified TGH (positive control); Sf9; homogenate from uninfected Sf9 cells, *wt*-TGH, homogenate from Sf9 cells infected with *wt*-TGH recombinant baculovirus, C390A, homogenate from Sf9 cells expressing mutant (C390A)

B. Same membrane immunoblotted with anti-TGH polyclonal antibodies.

C. Activity profile of cell homogenates from uninfected and infected Sf9 cells (both *wt*-TGH and mutants) using *p*-nitrophenyl laurate as substrate. This figure was provided by Dr. Mustafa Alam.

6.12 Discussion

As described above, there are five cysteine residues within TGH based on the deduced amino acid sequence of the cloned cDNA. Four of the cysteine residues are involved in intramolecular disulfide bonds, leaving one free cysteine residue (Cys 390). Here several possible roles of Cys 390 were investigated: (i) intermolecular disulfide bonding; (ii) acylation; (iii) involvement in catalysis. Three enzymatically active forms of a related porcine carboxylesterase (mono-, tri- and tetra-mer) were identified by size exclusion chromatography (Smialowski-Fleter *et al.*, 2002). The nature of the homo-oligomer interactions were not determined, although all three forms migrated as a monomer in reducing SDS-PAGE. It is unlikely that TGH exists as a covalent dimer *in vivo* since purified TGH from Sf9 cells migrates in SDS-PAGE at a "momomeric" molecular mass of 60 kDa in both reducing and non-reducing conditions.

The same porcine carboxylesterase that engages in homo-oligerizeration above, was also reported to undergo post-translational acylation (Smialowski-Fleter *et al.*, 2002). Experiments to determine whether Cys 390 in TGH was acylated were conducted because acylation can affect subcellular localization of proteins and their association with membranes. The substrate pool for TGH likely exists as lipid droplets within the ER (Figure 1-12). TGH has been shown to co-isolate with lipid droplets from liver (Lehner *et al.*, 1999). Acylation could facilitate interaction with the droplet surface. There is no known consensus amino acid sequence for thio-acylation of proteins, and therefore Cys 390 of TGH was a candidate for this modification. However, our data show that Cys 390 in TGH is not acylated and therefore interaction of TGH with lipid via this modification is unlikely. These data are also supported by the observation that rat (Lehner *et al.*, 1999; Lehner and Vance, 1999) and mouse (Dolinsky *et al.*, 2001) TGH do not have a corresponding free cysteine residue but can hydrolyze the same insoluble substrates.

Chapter 7:

Studies to determine if TGH is involved in protein-protein interactions

7.1 Introduction

Data from Chapter 6 demonstrated that TGH could not hydrolyze apoB associated lipids in isolated intracellular or secreted particles. The concept emerged that apoB may block access of TGH to lipids carried on these particles. Similarly, TGH may interact with proteins that facilitate or regulate the access of this enzyme to its endogenous substrate pools. Most readily hypothesized is that these TGH interacting proteins exist on the surface lipid droplets, thus moderating a tethering interaction at the surface of the droplet or presenting lipid such that it becomes available for hydrolysis. Experiments described in this Chapter were designed to determine if other proteins interact with TGH, and to subsequently isolate and identify them. Identification of TGH interacting proteins as factors known to operate in lipid metabolism may provide further mechanistic insights into how TGs are shuttled towards VLDL assembly.

7.2 Materials and Methods

7.2.1 Reagents

Sephacryl S-200 was purchased from Pharmacia, Uppsala, Sweden. Protein standards for characterizing the gel filtration column, pre-cast 4-15% polyacrylamide protein gels and electroporation cuvettes were purchased from Bio-Rad Laboratories, Mississauga, ON, Canada.

MTP antibodies were generously provided by Dr. David A. Gordon, Bristol-Myers Squibb. Calreticulin antibodies were a kind gift from Dr. Marek Michalak, University of Alberta. Phosphatidylethanolamine N-methyltransferase (PEMT) antibodies were a generous gift of Dr. Dennis E. Vance, University of Alberta. PDI antibodies were from Stressgen Biotechnologies, Victoria, BC, Canada. ApoE antibodies were purchased from BioDesign, Saco, ME. Anti-flag monoclonal antibody was from Stratagene, La Jolla, CA, while anti-flag monoclonal antibody covalently bound to agarose beads was from Sigma-Aldrich Oakville, ON, Canada. DOTAP transfection reagent was purchased from Roche Diagnostics, Indianapolis, IN, while ExGen 500 transfection reagent was from Fermentas Life Sciences (Burlingtion, ON, Canada).

DMEM lacking methionine and cysteine was from Invitrogen Canada (formerly Gibco-BRL Life Technologies) Burlington, ON, Canada. Pyruvate and glutamine levels in this media required supplementation (Sigma-Aldrich) to match the DMEM routinely employed in our cell culture systems. Easy Tag Expre^{35}S^{35}S Protein Labeling Mix containing [^{35}S] methionine and cysteine was from Perkin Elmer, Boston, MA. The homo-bifunctional protein cross-linker dimethyl 3,3´-dithiobispropionimidate (DTBP) was from Pierce Biotechnology, Rockford, IL.

7.2.2 Preparation of microsomes from rat liver and McArdle RH7777 cells

Microsomes from rat liver were isolated in a similar fashion as in Section 3.2.2. A male Sprague-Dawley rat of approximately 125 g body weight was deeply anesthetized. The abdominal cavity was opened, and intestines displaced to reveal the lower aorta. Using a syringe, the rat was exsanguinated via the abdominal aorta. The liver was immediately removed and disrupted with scissors to generate pieces 3 mm in diameter or smaller. The resulting preparation was combined with 70 mL of ice-cold buffer containing 250 mM sucrose, 50 mM Tris pH 7.4, 5 mM EDTA and 1 mM benzamidine. Further disruption was accomplished using a Waring blender for 1 min at high speed followed by 30 sec at low speed. The resulting suspension was passed through cheesecloth, and then centrifuged at 7,800 x*g* for 10 min at 4°C. The supernatant was subjected to ultracentrifugation at 106,000 x*g* for 1 h at 4°C to obtain microsomal membranes in the pellet. The pellet was resuspended in 20 mL of TBS containing 1 mM benzamidine using 10 strokes on a Potter-Elvehjem apparatus, producing an opaque solution. The volume was adjusted with TBS-benzamidine such that the final concentration of protein was 10 mg/mL. In preparation for gel filtration chromatography, 1 mL of 10% Triton X-100 was combined with 9 mL of the microsomal preparation and allowed to sit on ice for 50 min with inversions after 20 and 40 min. Detergent insoluble debris was removed from this preparation by centrifugation at 2,000 x*g* for 5 min at 4°C

Microsomess from McArdle RH7777 cells stably expressing human or rat TGH were prepared from six 100 mm cell culture dishes of approximately 75%

confluence. Cells were harvested in sucrose buffered saline (250 mM sucrose, 50 mM Tris pH 7.4, 5 mM EDTA), disrupted by sonication and microsomes prepared via centrifugation as they were from rat liver homogenates described above. Microsomes from McArdle RH7777 cells were also homogenized in TBS, adjusted to 10 mg protein/mL, then brought to 1% Triton X-100 for solubilization. Detergent insoluble material was removed by centrifugation as above.

7.2.3 Detergent solubility and membrane topology of TGH in rat liver microsomes

Microsomes from rat liver prepared as above, resuspended in TBS containing Complete protease inhibitor cocktail and adjusted to 10 mg protein/mL. Four aliquots of 20 µL each were dispensed. The aliquots received 480 µL of TBS, TBS with 1% Triton X-100, TBS with 0.1% CHAPS or 0.2 M sodium carbonate pH 11.5. Samples were maintained on ice for 15 min, then centrifuged at 425,000 x*g* for 45 min at 4°C. The resulting pellets were resuspended in 500 µL of 50 mM Tris pH 8. An additional 45 µL of 1 M Tris pH 8 was delivered to the supernatant from sodium carbonate treatments to adjust the pH. Proteins in 45 µL of pellet and supernatant fractions were separated by SDS-PAGE (10%), transferred to nitrocellulose membranes and probed by immunoblot for the distribution of TGH, PEMT and calreticulin.

7.2.4 Preparation of a Sephacryl S-200 gel filtration column

Approximately 200 mL of Sephacryl S-200 were washed twice with TBS containing 1% Triton X-100 to remove the solvent supplied for packaging and storage. The slurry was introduced to a column 50 cm long with 1 cm inside diameter. TBS with 1% Triton X-100 was allowed to flow through the column at 0.5 mL/min for approximately 15 h to thoroughly wash and pack the material. Commercially prepared protein standards (Bio-Rad) were passed through the column, also at a flow rate of 0.5 mL/min. An automated fraction collector was used to collect 2 mL fractions. Every second fraction was analyzed for protein

content using the bicinchoninic acid protein assay reagent (Pierce Biotechnology, Rockford, IL) using BSA as a standard according to the manufacturer's protocol.

7.2.5 Gel filtration of Triton X-100 solubilized microsomes

Triton X-100 solubilized microsomes were passed through the Sephacryl S-200 column (Section 2.2.2) at a flow rate of 0.5 mL/min using TBS containing 1% Triton X-100 as the elution buffer; 2 mL fractions were collected. Protein content was determined in the fractions; lipase activity in 20 µL of each fraction was analyzed in assays using *p*-nitrophenyl laurate as a substrate as per Section 2.9. Proteins in 45 µL of each fraction were separated by SDS-PAGE (12%), and transferred to nitrocellulose membranes. Immunoblots were performed using anti-TGH antibodies. Further characterization of fractions determined to contain lipase activity and TGH were performed. Pre-cast 4-15% polyacrylamide gels were used to separate proteins in these fractions in both native and denaturing conditions. Native PAGE simply lacked SDS and β-mercaptoethanol from the sample treatment buffer and lacked SDS from the running buffer. After electrophoresis and transfer of the proteins, the resulting nitrocellulose membranes were cut in order to immunoblot for the presence of TGH, MTP and PDI simultaneously in samples that had been handled equivalently.

7.2.6 Electroporation of primary rat hepatocytes

Electroporation of primary rat hepatocytes was performed based on previously published protocols (Morimura *et al.*, 1992; Paquereau and Le Cam, 1992; Yamamoto *et al.*, 2001; Yao *et al.*, 2002). Rat hepatocytes were isolated from the animals by collagenase perfusion of the liver as per the procedure described in Section 2.5.3. Aliquots of $1.5x10^7$-$2.0x10^7$ cells were dispensed into 15 mL conical tubes. Cells were pelleted by centrifugation, resuspended in PBS, DMEM or DMEM containing 10% FBS and combined with 15-20 µg of pCI-neo plasmid DNA ($1x10^6$ cells/µg DNA) carrying the flag-epitope tagged human TGH cDNA developed in Section 4.6.4. The cells and plasmid DNA were maintained on ice for 10-15 min. Electroporations were performed on a BTX ECM630

Electrocell Manipulator using electroporation cuvettes with a 2 mm gap. The apparatus was used to deliver 1,000 μF capacitance, 200 volts and 75 ohms, which typically required 7-10 msec. Immediately after electroporation, cells were resuspended in DMEM containing 10% FBS and plated on collagen coated tissue culture dishes.

7.2.7 Immunoprecipitation of TGH from rat hepatocytes after [^{35}S] protein labeling and protein cross-linking

After electroporation and plating, cells were incubated for 4 h then media were changed in order to remove non-viable cells and debris. Incubation was continued overnight. Media were removed via aspiration, cells washed with PBS and incubation continued in cysteine and methionine free DMEM for 1 h. Media were again removed by aspiration and replaced with cysteine and methionine free DMEM supplemented with 50 μCi/mL (2 mL/60 mm dish) of Easy Tag Expre^{35}S^{35}S Protein Labeling Mix for 30-60 min in order to radio-label newly synthesized cellular proteins. Perkin Elmer estimates the distribution of [^{35}S] labeled components is routinely 73% [^{35}S] methionine and 22% [^{35}S] cysteine in their mixture. Media were replaced with regular DMEM containing 10% FBS for 15-30 min. Some dishes of cells (either [^{35}S] labeled or not) were then washed with PBS and incubated with 8 mM of the homo-bifunctional protein cross-linker DTBP in PBS for 50 min at room temperature. Following aspiration of this cocktail, DTBP still associated with the cells was quenched via incubation in TBS for 5-10 min. Cells were harvested in TBS containing 1% Triton X-100 and Complete protease inhibitor cocktail (Roche Diagnostics). Detergent lysis was allowed to precede over 2 h on an end-over-end rotating rack at 4°C. Samples were centrifuged at 1,000 x*g* for 2 min to pellet unlysed cells and detergent insoluble debris. Aliquots of the supernatants were frozen, while the remainder of the samples were transferred to clean tubes and combined with an equal volume of TBS containing Complete protease inhibitor cocktail. The addition reduced the detergent concentration to 0.5% Triton X-100 (total volume of 800 μL). Following this, 30 μL of anti-flag antibody attached to agarose beads were

added, and the slurry allowed to incubate for 4 h at 4°C on a rotating rack. Beads were washed twice with TBS containing 0.5% Triton X-100, and then once with TBS. Denaturing electrophoresis buffer with was added to the IP beads as well as aliquots of cell homogenates. Proteins in the samples were separated by SDS-PAGE and transferred to nitrocellulose. Membranes were exposed to phosphorimaging screens that were then analyzed on a Bio-Rad Molecular Imager FX Phosphorimager. Immunoblots were performed with the primary antibodies indicated in figure legends.

7.3 Results

7.3.1 Membrane topology and detergent solubility of rat liver TGH

Evidence of TGH binding partners in rat liver microsomes was initially suggested by assays of TGH solubility in sodium carbonate and in various detergents. Sodium carbonate pH 11.5 is a commonly used agent to release lumenal and peripherally associated membrane proteins, while 0.1% CHAPS will partially disrupt membranes and release lumenal contents without solubilizing integral membrane proteins. The 1% Triton X-100 treatment will dissolve membrane lipids and solubilize transmembrane proteins, while maintaining stable protein-protein interactions. These conditions were used to treat rat liver microsomes, followed by analysis of the solubility of PEMT, calreticulin and TGH via immunoblot. PEMT is a transmembrane protein of the ER (Vance *et al.*, 1997), while calreticulin is a soluble protein chaperone within the lumen of this compartment, and as such, is involved in protein-protein interactions (Ellgaard *et al.*, 1999). As expected for soluble proteins, 0.2 M sodium carbonate pH 11.5 and 0.1% CHAPS caused release of some calreticulin and TGH from the pelleted fraction compared to control incubations, while maintaining the association of PEMT with the membranes (Figure 7-1). Treatment with 1% Triton X-100 released the transmembrane protein PEMT into the soluble fraction, however, a population of both calreticulin and TGH remained in the pellet. The result indicates that like calreticulin, a portion of TGH remained in the pelleted fraction

Figure 7-1 Resistance of TGH to detergent solubilization and membrane topology. Microsomes were isolated from rat liver, then resuspended in 100 μL of TBS. Four aliquots were prepared from this liquor and incubated in 0.2 M sodium carbonate (Na_2CO_3) pH 11.5, TBS containing 0.1% CHAPS or 1% Triton X-100 or buffer alone (CTRL) for 15 min on ice. All preparations were then centrifuged at 425,000 x*g*. The pellets were resuspended in a volume of TBS equal to that found in supernatants. Proteins in aliquots of the supernatants (S) and pellets (P) from all treatment groups were separated by SDS-PAGE, transferred to nitrocellulose membranes and probed for TGH, phosphatidylethanolamine *N*-methyltransferase (PEMT) or calreticulin (CR) using respective antibodies.

due to its participation in protein complexes. Alternately, a portion of TGH may have become involved in aggregates during sample preparation.

7.3.2 Gel filtration of Triton X-100 solubilized rat liver microsomes

Studies to isolate and identify TGH interacting proteins were initiated using Sephacryl S-200 gel filtration chromatography. The column was initially characterized using protein standards (Figure 7-2 A). These proteins separated over approximately 40 fractions, with the first 10 representing the void volume. The column was used subsequently for the separation of proteins in Triton X-100 solubilized rat liver microsomes. Again, the proteins contained in this preparation separated over approximately 40 fractions (Figure 7-2 B). Lipase activity was then probed in assays utilizing *p*-nitrophenyl laurate as a substrate. As shown in Figure 7-2 C, two major peaks of activity eluted. Only background levels of lipase activity were observed in remaining fractions. Immunoblots for TGH demonstrated that only fractions containing lipase activity contained TGH (Figure 7-2 D). The intensity of the TGH band in the immunoblot shown in Figure 7-2 D mimics the amount of lipase activity found in those fractions. That is, fractions 16 and 20 had the most lipase activity (Figure 7-2 C), and the most intense bands for TGH in the immunoblot (Figure 7-2 D). Hence two populations of TGH were eluted from the gel filtration column: one in the range of 165 kDa, and another in the range of 60 kDa (the size of the monomer). Native PAGE of fractions 16 and 20, followed by anti-TGH immunoblot (Figure 7-3 A) showed a difference in electrophoretic mobility, again indicating that rat TGH is at least partly found in protein complexes that are stable in 1% Triton X-100. The presence of MTP and PDI were also probed in these fractions by immunoblot after denaturing SDS-PAGE (Figure 7-3 B). The presence of both of these proteins in fraction 16, but not fraction 20 suggests that these two species could potentially be involved in a multi-protein complex with TGH.

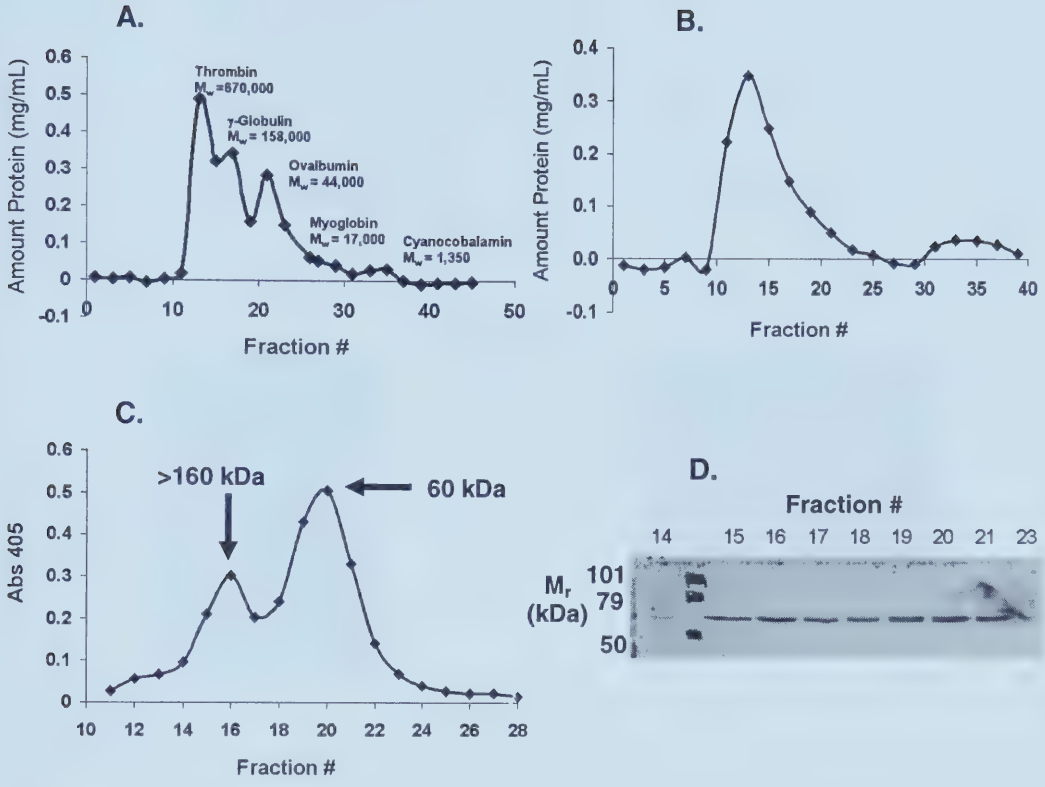


Figure 7-2 Gel filtration chromatography of rat liver microsomes produces two fractions with lipase activity that both contain TGH. Microsomes were isolated from rat liver, then solubilized in TBS containing 1% Triton X-100. Proteins in the microsomes were then separated by size on a gel filtration column containing Sephacryl S-200 as described in Materials and Methods.

A. Commercially prepared protein standards were separated on the gel filtration column over approximately 40 fractions.

B. Proteins in solubilized rat liver microsomes are also separated over approximately 40 fractions.

C. Lipase activity was assessed in each fraction using *p*-nitrophenyl laurate as a substrate. Data are represented as the spectrophotometric absorbance at 405 nm (Abs 405) per fraction.

D. Immunoblot for TGH in Sephacryl S-200 column fractions. Proteins from equal volumes of the indicated fractions (fractions containing lipase activity) were separated by SDS-PAGE, transferred to a nitrocellulose membrane, then probed from the presence of TGH by immunoblot.

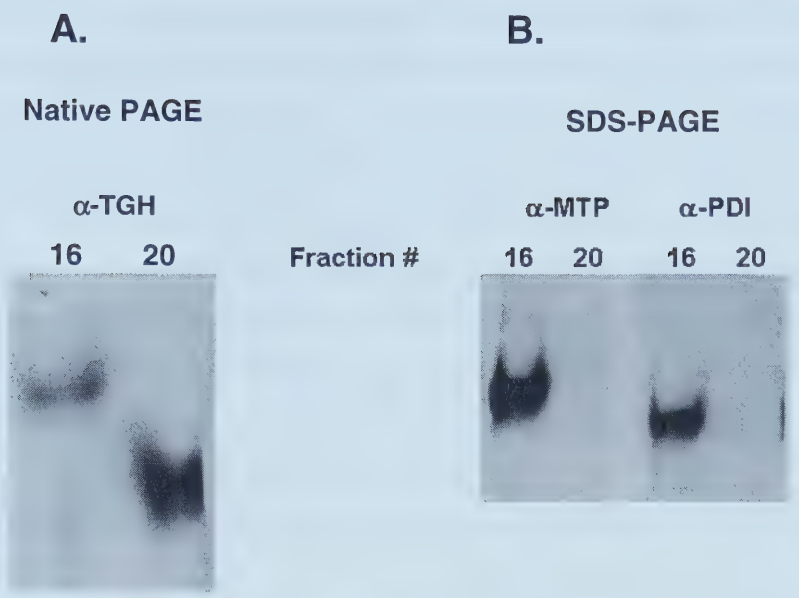


Figure 7-3 Characterization of TGH containing gel filtration fractions by electrophoresis and immunoblot. Gel filtration experiments were performed to isolate 2 populations of TGH in fractions 16 (high molecular weight) and 20 (approximate size of monomer based on elution of standards).
A. Native PAGE was performed on fractions 16 and 20, proteins were transferred to nitrocellulose membranes and probed for TGH via immunoblot.
B. SDS-PAGE was performed on fractions 16 and 20, proteins were transferred to nitrocellulose membranes and probed for microsomal triglyceride transfer protein (MTP) and protein disulfide isomerase (PDI).

7.3.3 Gel filtration of Triton X-100 solubilized microsomes isolated from TGH stably transfected McArdle RH7777 cells

Similar gel filtration experiments using microsomes isolated from McArdle RH7777 cells stably expressing either rat or human TGH indicate this enzyme does not form oligomeric protein complexes in this cell line. As shown in Figure 7-4, only one peak of lipolytic activity arises in the resulting fractions. Potentially, this cell line does not possess the putative binding partners. The results from McArdle RH7777 cells also suggest that the higher molecular weigh complex is not a homo-oligomer, as this would likely not require other factors to assemble. Our polyclonal anti-TGH antibodies are inefficient or incapable of IP of rat or human TGH, therefore an epitope tag was required to facilitate a co-immunoisolation strategy. Attempts to isolate TGH binding partner(s) from McArdle RH7777 cells via co-immunoprecipitation, SDS-PAGE and silver staining confirmed that these complexes are not assembled in this cell line.

7.3.4 Expression of flag-epitope tagged TGH in primary rat hepatocytes by electroporation

Transfection of primary rat hepatocytes with a TGH construct that included a flag-epitope tag was attempted using cationic liposome based transfection reagents, including Lipofectamine2000, DOTAP and ExGen 500. Production of immuno-detectable quantities of TGH in cell lysates of transfected cells, however, was not accomplished. In order to increase transfection efficiencies, electroporation of rat hepatocytes was executed. Electroporations were performed in PBS, DMEM, or DMEM with 10% FBS. Similar cell survival rates based on trypan blue exclusion were found using all three electroporation media, although reducing the time between isolating the cells and performing the electroporation enhanced cell survival rates. All three electroporation media resulted in isolation of similar amounts of flag-epitope tagged TGH by IP (Figure 7-5 A and B). Therefore, subsequent electroporations were performed in DMEM with 10% FBS.

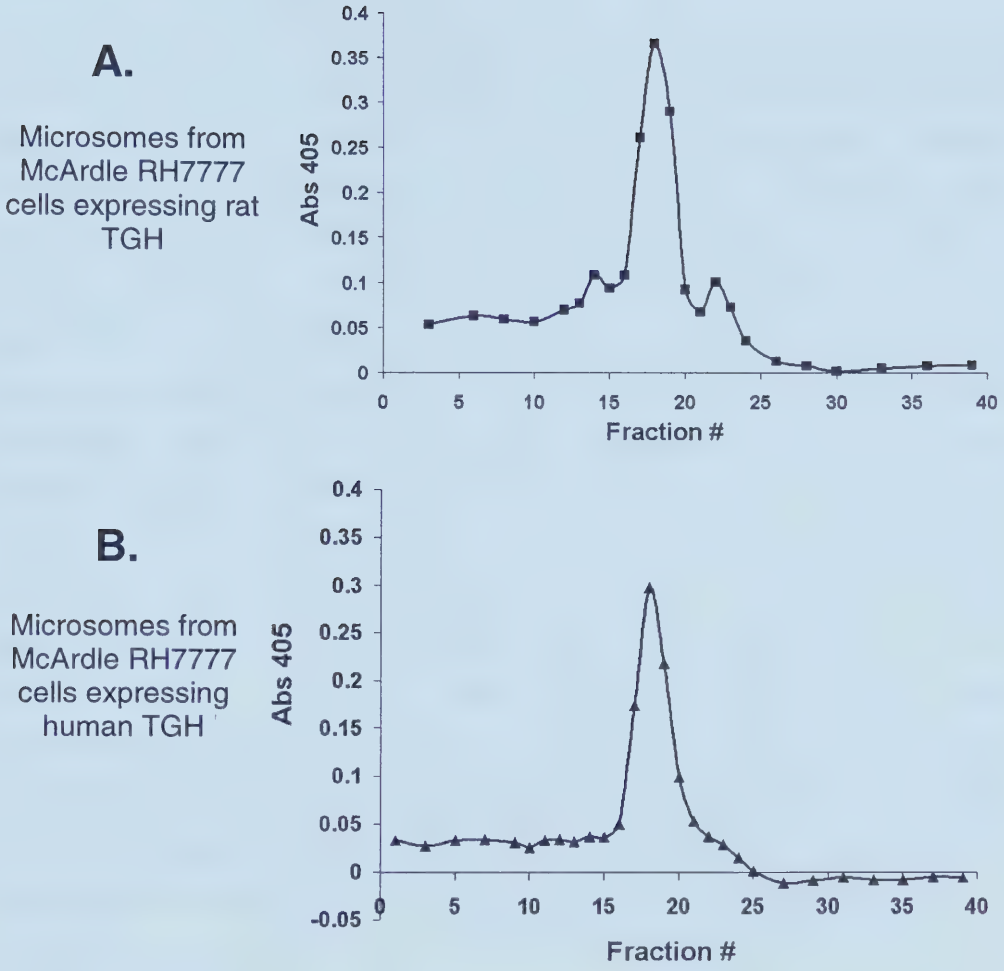


Figure 7-4 Lipase activity in microsomes isolated from stably transfected McArdle RH7777 cells. Microsomes were isolated from McArdle RH7777 cells that were stably transfected with rat TGH (**A.**) or human TGH (**B.**) via centrifugation. As in experiments with rat liver microsomes (Figure 7-2), microsomes from McArdle RH7777 cells were solubilized in TBS supplemented with Triton X-100, then proteins separated by size on a gel filtration column and activity assays performed using *p*-nitrophenyl laurate as a substrate. Data are shown as the spectrophotometric absorbance at 405 nm (Abs 405) resulting from liberation of *p*-nitrophenol in the respective fractions.

7.3.5 Protein cross-linking and immunoprecipitation of TGH from electroporated and radio-labeled rat hepatocytes

As shown in Figure 7-5, electroporation provided a method to express, then isolate TGH from primary rat hepatocytes. Experiments to validate that other proteins were co-isolating with TGH were then engaged. Following electroporation of primary rat hepatocytes with plasmid encoding flag-epitope tagged TGH, newly synthesized proteins were radio-labeled as described in Materials and Methods. Cells were then treated with the protein cross-linking agent DTBP, and flag-epitope tagged TGH isolated by IP. Inclusion of the protein cross-linking agent stabilizes an interaction with TGH binding partners for co-isolation. Radio-labeling cellular proteins with [^{35}S] methionine and cysteine provided a sensitive method of detection (phosphorimaging) and also eliminated obstructions provided by fragments of the immunoprecipitating antibodies upon attempts to visualize co-isolating species. DTBP can be cleaved by reducing agents. Therefore, cross-linked species migrated in SDS-PAGE as the free protein following treatment with denaturing electrophoresis buffer. Phosphorimaging in Figure 7-6 A shows that numerous proteins can be cross-linked and immuno-isolated with flag-epitope tagged TGH. Immunoblots verified that TGH was isolated from electroporated cells (Figure 7-6 B). Immunoblots for apoE revealed that this protein can be co-isolated with TGH (Figure 7-6 C).

7.4 Discussion

It is recognized that most other TG lipases identified to date interact with a "cofactor" with functional consequences. As described in Section 1.5.2, perilipins are found on the surface of lipid droplets in adipose tissue and are described as providing a barrier to HSL, thus preventing its access to substrate. Upon phosphorylation of both HSL and perilipin by PKA, an interaction is promoted, HSL translocates to the surface of the lipid droplet and TG lipolysis is enhanced. ApoCII is considered a specific activator or cofactor of LpL, although attenuated lipase activity is still observed in the absence of apoCII (Mead *et al.*, 2002).

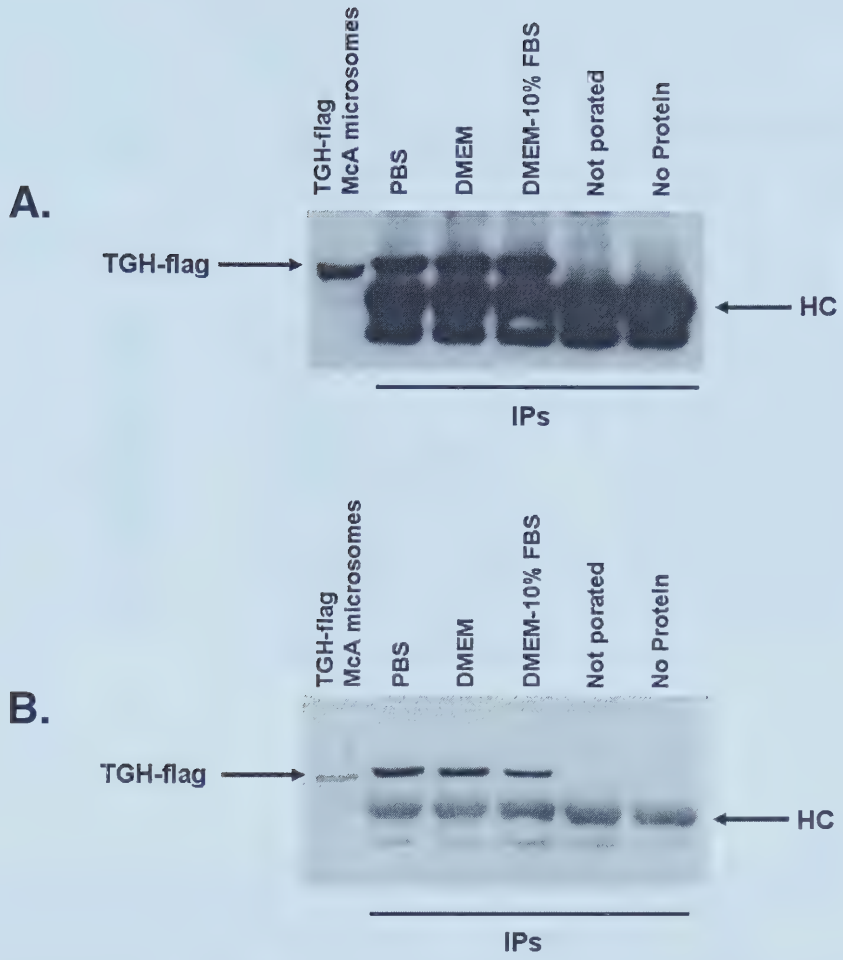


Figure 7-5 Flag-epitope tagged TGH expression in primary rat hepatocytes via electroporation. The pCI-neo plasmid DNA coding for flag-epitope tagged TGH was electroporated into primary rat hepatocytes as described in Materials and Methods. Electroporation was performed in a variety of media, including PBS, DMEM and DMEM supplemented with 10% FBS. Following electroporation, cells were grown overnight, harvested, and TGH isolated by immunoprecipitation (IP) from cell lysates using anti-flag antibody. Immuno-isolated proteins were dissociated from IP beads in denaturing electrophoresis buffer, separated by SDS-PAGE, transferred to nitrocellulose membranes and probed for flag-epitope tagged TGH using anti-flag (panel **A.**) or anti-TGH (panel **B.**) antibodies. Controls included IPs from hepatocytes that were not electroporated (not porated) or did not contain a cell lysate (no protein). Microsomes isolated from McArdle RH7777 cells (McA) stably expressing flag-epitope tagged TGH were also included on the gels to identify the TGH-flag band in immunoblots. Also indicated is the heavy chain (HC) of the immunoprecipitating antibody.

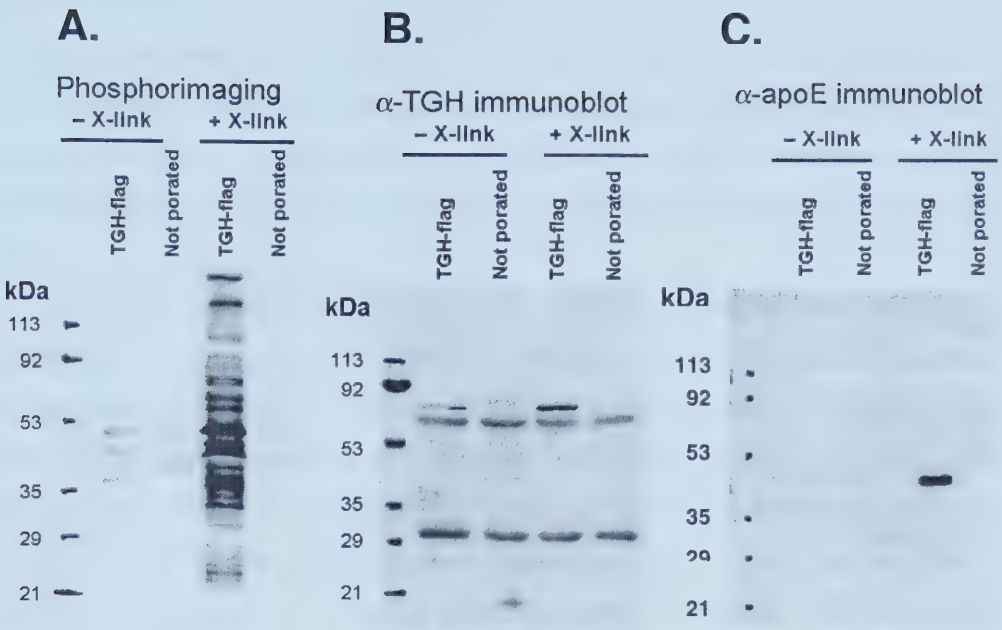


Figure 7-6 Co-isolation of proteins from primary rat hepatocytes with flag-epitope tagged TGH. A plasmid encoding flag-epitope tagged TGH was expressed in primary rat hepatocytes via electroporation. After incubating overnight, media were changed to DMEM lacking methionine and cysteine. Newly synthesized proteins were then radio-labeled with [^{35}S] methionine and cysteine. Subsequently, some cells (electroporated or not) were incubated with the protein cross-linking agent dimethyl 3,3´-dithiobispropionimidate (DTBP; indicated as X-link). Cells were harvested, and immunoprecipitation (IP) of flag-epitope tagged TGH was performed using anti-flag antibodies. Immuno-isolated proteins were separated from IP beads using denaturing electrophoresis buffer. Proteins were resolved by SDS-PAGE (4-15% gradient) and transferred to nitrocellulose membranes. Lanes from samples prepared from cells that were subjected to electroporation are indicated as TGH-flag. Membranes were exposed to phosphorimaging screens to detect radio-labeled proteins (panel **A.**) and probed for TGH (panel **B.**) and apolipoprotein E (apoE; panel **C.**) via immunoblot using respective antibodies.

Pancreatic triglyceride lipase is the principle enzyme in the digestive tract that hydrolyzes dietary TGs for absorption. Activation of enzymatic activity is achieved upon binding of colipase, which is also a protein produced and secreted by the pancreas (Hui and Howles, 2005; Lowe, 1994). BSDL is another lipolytic enzyme found in pancreatic secretions with broad substrate specificity (Lombardo, 2001). It is activated by binding of primary bile salts in the duodenal lumen.

Considering the precedents for TG lipases interacting with cofactors to mediate various aspects of their function, it is likely that TGH is also influenced by a binding partner(s). Potentially, TGH interacting proteins are involved in a regulatory mechanism for TG mobilization from storage pools. Several lines of evidence presented herein have indicated that TGH interacts with proteins in the rat liver. Gel filtration experiments indicate that TGH containing multi-protein complexes are present in the rat liver, but do not form in TGH transfected McArdle RH7777 cells. Presumably a factor involved in forming or maintaining this complex is lacking in that cell line. Anti-TGH polyclonal antibodies developed in our laboratory were not capable of efficient immunoprecipitations, fostering the employment of epitope tags. Experiments utilizing electroporation of primary rat hepatocytes with epitope tagged TGH indicated that several proteins can be cross-linked to TGH, again suggesting their interaction. One of these was identified as apoE (Figure 7-6 C). It is well documented that apoE null mice have a pro-atherogenic lipoprotein profile, and are a commonly used model for investigation of this disease. The foundation for the atherosclerotic predisposition is a decreased rate of clearance of apoB containing particles from plasma, as lipid bound apoE can interact with various cell surface receptors and mediate endocytosis. Intriguingly, isolated hepatocytes from apoE null mice have a decreased rate of VLDL secretion (Kuipers *et al.*, 1997). Potentially, apoE may exist on lipid droplets present in the hepatic ER lumen and fascilitate an interaction between TGH and this substrate pool. Demonstration of apoE on hepatic lipid droplets is the focus of current investigation in our laboratory. Further confirmation of the apoE – TGH interaction proved difficult to achieve.

The experimental design of the electroporations may be further improved, as a human TGH construct was expressed in rat hepatocytes. Rat and human TGH are approximately 93% identical at the amino acid level (Dolinsky *et al.*, 2004b), however, the most relevant rat TGH binding proteins may not interact with human TGH.

In conclusion, the data presented herein demonstrate that TGH exists in a multi-protein complex(es) in rat liver that is at least partially stable in 1% Triton X-100. Identifying TGH binding partners with known functions in lipid metabolism may reveal a more detailed mechanism of how TGs are trafficked towards VLDL assembly. Identification of these proteins and confirmation that apoE is a *bona fide* TGH interacting protein requires further investigation. Strategies and reagents that simplify and improve the existing experimental designs have been developed and are described as future directions in Chapter 8.

Chapter 8:

Summary, concluding remarks and future directions

8.1 Summary

In Chapter 1, the potential for neutral lipids to contribute to disease was described, exposing inadequacies in the understanding of neutral lipid trafficking as well as disturbing trends in the increasing prevalence of disorders involving neutral lipids, such as obesity, diabetes and cardiovascular disease. A brief review of neutral lipid metabolism followed, with an emphasis on proteins involved in TG synthesis, storage, lipolysis and secretion. Special attention was given to the contribution of triacylglycerol hydrolase (TGH) and its role in the liver to hydrolyze stored TG, thus mobilizing it for very low density lipoprotein (VLDL) assembly and secretion. Because high levels of plasma VLDL and TG are risk factors for cardiovascular disease, a pharmacological relevance for TGH (inhibition) was revealed. Further study of TGH could provide the basis for future therapeutic approaches that may alleviate the risk of atherosclerosis. Details regarding research that revolved around this enzyme was the unifying theme for the remainder of the Thesis. In summary, by examining this enzyme, a better understanding of neutral lipid metabolism in general has been accomplished.

The characterization of TGH is a major advance, since relatively little was known about intracellular TG lipases with the exception of HSL. Substantial progress has been made towards our understanding of how TGH operates in the liver and its role in VLDL assembly. Further investigations into the function and regulation of this enzyme could better establish a link between TGH and the maintenance of the correct energy balance within an organism.

Chapter 2 described details of techniques that were routinely employed during the course of these studies. Topic specific applications appear in the relevant Chapters.

In Chapter 3, inhibition of lipolysis by the irreversible serine esterase inhibitor E600 and by a specific TGH inhibitor 4,4,4-trifluoro-2-[2-(3-methylphenyl)hydrozono]-1-(2-thienyl)butane-1,3-dione reduced the secretion of TG and apoB by primary rat hepatocytes and TGH stably transfected McArdle RH7777 cells. The inhibitors had little effect on endogenous lipolytic activity in empty plasmid transfected McArdle RH7777 cell microsomes, indicating the

expressed TGH became the most active neutral lipase present. As well, decreases in cellular CE were observed after incubations with the inhibitors, indicating that TGH activity may supply fatty acids as substrates for ACAT. In addition, acute feeding of hamsters (3 days) with the specific inhibitor resulted in significant decreases in plasma TG, VLDL-cholesterol and apoB. HDL cholesterol was not significantly altered. Collectively, the evidence demonstrates that TGH is involved in the lipolysis of stored TG, which then becomes available for secretion. These findings validate TGH as a pharmacological target for cardiovascular disease, as inhibition could reduce the levels of plasma LDL, a major contributor to advance of atherosclerosis (see Section 1.6)

Data presented in Chapter 4 focused on subcellular localization of TGH. The investigations showed that deletion of the C-terminal -HIEL amino acid sequence from TGH (termed ΔR-TGH) resulted in secretion of the expressed protein into the culture media of McArdle RH7777 and Cos-7 cells without loss of specific enzyme activity. These results agreed with the published observations of others, in that the -HXEL sequence is a functional ER-retrieval sequence in mammalian cells (Robbi and Beaufay, 1991) and is the element responsible for retaining TGH within the ER lumen. Immunogold electron microscopy of McArdle RH7777 cells transfected with the TGH cDNA showed that TGH was associated with ER membranes, but was not detected in the cytosol, Golgi stacks, mitochondria or nuclei. TGH is not evenly distributed throughout the ER, but rather is found clustered in cisternae surrounding mitochondria. The hypothesis developed in which TGH is localized to regions specialized in VLDL production, and this localization facilitates access to its substrate or confers enhanced ability to transfer products of lipolysis towards assembly with nascent apoB. Mutation of the unusual (deviation from consensus) C-terminal ER retrieval sequence to the consensus sequence found in animal cells (-KDEL), and expression in McArdle RH7777 cells resulted in reduced ability of the mutant to direct TG towards secretion. Confocal immunofluorescence microscopy revealed that *wt*-TGH does not completely colocalize with other soluble ER proteins with the -KDEL ER retrieval sequence. Further, *wt*-TGH was present in compartments

near the cell periphery that are devoid of the KDEL-TGH mutant. These observations suggest that the unusual C-terminal sequence is not only responsible for retention of the protein in the ER, but also directs TGH to a unique region of the compartment for efficient participation in VLDL assembly. Neutral lipid storage droplets are usually found in the cell periphery, so the proximity to peripherally localized *wt*-TGH may be the foundation for an enhanced ability of the *wild type* enzyme to mobilize stored TG for VLDL assembly and secretion versus KDEL-TGH. The mechanism for delivery of *wt*-TGH to this region may involve a receptor other than the KDEL-receptor that delivers cargo to a distinct region of the ER, or alternately may involve a protein resident to the ER that can interact with the -HIEL sequence and escort it to a new location. Strategies to isolate the factor involved in this process have been developed, and are the focus of current investigation in our laboratory. Synthetic 14 amino acid peptides matching the C-terminal sequence of TGH have been designed. They can be bound to a solid support in order to isolate proteins that interact by affinity chromatography. Additionally, the peptides can be used to generate antibodies that may be successful in immuno-isolations of TGH and interacting proteins.

Chapter 5 focused on post-translational modifications to TGH. This enzyme is a glycoprotein (Alam *et al.*, 2002b), and a lone glycosylation event is predicted at Asn 79. *N*-glycosylation occurs in the lumen of the ER, so modification of TGH in this manner is further support for its localization in this compartment. In Chapter 5, tunicamycin treatment reduced glycosylation of TGH by at least 60%, but did not result in reduced lipolytic activity. Subsequently, the glycosylation site was mutated, and specific enzyme activity was not altered. This was somewhat surprising as *N*-glycosylation mediates interactions with protein chaperones and facilitates proper folding of many proteins manufactured in the ER (Ellgaard *et al.*, 1999).

Investigation of the potential phosphorylation or acylation of TGH were also presented in Chapter 5. Phosphorylation of several other lipases and carboxylesterases is known to occur and induce functional alterations such as

changes in enzymatic activity and localization (see Section 5.8). It is known that insulin reduces hepatic VLDL secretion, and particular attention was given to the potential that insulin mediates this effect via phosphorylation of TGH through a signal transduction cascade. Reduced mobilization of stored lipids through modification of TGH would reduce VLDL secretion, as indicated by the results of Chapter 3. Similarly, transient acylation of TGH may increase its affinity for hydrophobic surfaces, including the surface of lipid droplets that represent the TGH substrate pool. Hence acylation of TGH may alter the availability of TG for secretion. Neither phosphorylation nor acylation of TGH was observed; therefore regulation or modulation of TGH activity operates independently of these mechanisms.

In Chapter 6, the ability of TGH to hydrolyze lipids associated with apoB containing particles was examined. Both secreted and intracellular particles were assayed, and neither served as a substrate for TGH while a microbial lipase efficiently hydrolyzed the neutral lipids present. These findings provide provocative mechanistic insights into the function of TGH. They suggest a system for vectored movement of TG from storage pools to VLDL assembly, as once associated with apoB, TGH can no longer access this substrate. In addition, TGH cannot participate in "delipidation" of apoB containing particles targeted for degradation. Degradation of apoB likely involves retrotranslocation from the ER, which should require stripping the apoB associated lipids. This mechanism would be independent of TGH.

Chapter 7 presents data indicating TGH interacts with other proteins in a hetero-oligomeric complex. Evidence of a TGH containing complex was found in rat liver microsomes, but not in microsomes isolated from TGH transfected McArdle RH7777 cells. It is likely that this cell line lacks the TGH binding partner(s) or a factor involved in the assembly of the complex. Experiments designed to co-isolate TGH binding partners were performed with rat hepatocytes, and apoE was identified after protein cross-linking and IP. Verification that apoE participates in a TGH containing protein complex remains to be demonstrated. Because current TGH antibodies do not efficiently IP,

epitope tags and protein expression systems were required. The expression of flag-epitope tagged TGH in primary hepatocytes in Chapter 7 may have been flawed in that a plasmid encoding a human TGH construct was electroporated into rat cells. The rat TGH binding partner(s) may not bind human TGH. Several reagents and strategies were developed for use in future experiments. Poly-histidine tagged mouse TGH constructs were developed. The constructs include six histidine residues immediately prior to the ER retrieval sequence, or in replacement of the ER retrieval sequence (secreted protein). Following stable production in McArdle RH7777 cells, either protein can be purified in native conditions on a resin containing nickel via affinity chromatography. The purified protein can be used to generate antibodies that are capable of immuno-isolating TGH and its binding partner(s). As indicated above, peptides have been designed to prepare new polyclonal antibodies towards the C-terminal region of TGH. These antibodies may be more efficient in TGH immuno-isolations. Binding partners may be co-isolated. Alternately, purified poly-histidine tagged TGH can be incubated with mouse or rat liver homogenates. Tagged TGH may engage in typical protein-protein interactions, and the entire complex isolated via affinity chromatography. Finally, the poly-histidine tagged TGH construct that includes the ER retrieval sequence can be electroporated into primary mouse or rat hepatocytes, then the higher molecular weight complex isolated again via a nickel containing resin. Once isolated and visualized on polyacrylamide gels, TGH co-isolating species can be identified by mass spectrometry. Identification of TGH binding partners as proteins known to operate in lipid metabolism may provide significant insights into TGH mediated contributions to VLDL assembly.

TGH hydrolyzes insoluble carboxylic esters at the lipid / water interface, however, the mechanism by which the enzyme adsorbs to lipid droplets is unclear. A model has been presented (Figure 1-12) in which the TGH substrate pool in hepatocytes exists in an ER lumenal lipid droplet. TG-rich lipid droplets that are free of apoB are present in the ER lumen, and their formation requires the activity of MTP (see Section 1.3.3). Lipids associated with apoB are not substrates for TGH, suggesting a barrier function of apoB towards TGH-mediated

lipolysis. Potentially other lipid droplet coat protein(s) in the ER lumen of hepatocytes, such as apolipoproteins, modulate TGH access to this substrate pool and are involved in regulating the mobilization of the TG from these pools.

Intracellular neutral lipid droplets are surrounded by a monolayer of phospholipids and surface proteins (reviewed in (Londos *et al.*, 1999b; Murphy, 2001)). It is unclear if these surface proteins serve a structural role to maintain the lipid droplet integrity, prevent fusion with any adjacent lipophilic surfaces or have a functional role as docking sites for lipogenic or lipolytic enzymes. Recent evidence described in Section 1.5.2 has clarified the importance of lipid droplet coat proteins for regulating the association of HSL and mobilizing TG in adipose tissue. A similar mechanism for docking TGH to lipid droplets may be operating in the liver. Subcellular fractionation of rat liver homogenates revealed that TGH was present in the floating fat droplet (Lehner *et al.*, 1999). It is intriguing to speculate that a hepatic TGH binding partner may be a lipid droplet coat protein in the ER lumen that regulates association of this lipase.

8.2 Future directions

Details regarding how TGH engages lipid substrates remain obscure. It has been proposed (Au-Young and Fielding, 1992) that a neutral lipid-binding domain (NLBD), found in a variety of proteins that interact with or metabolize neutral lipids, could potentially promote this interaction. Au-Young and Fielding (Au-Young and Fielding, 1992) proposed a NLBD in human plasma cholesteryl ester transfer protein. This putative lipid-binding octapeptide forms an amphipathic α-helix with the consensus sequence C/FLXLXXXn (where n is a non-polar residue and X is any amino acid except proline). By sequence alignment, a putative NLBD motif was identified in TGH (Figure 1-7). The NLBD is contained in an α-helix adjacent to a hydrophobic entry pocket leading to buried catalytic residues (Figure 1-9). Thus the NLBD may regulate or facilitate interaction with hydrophobic surfaces such as membranes or lipid droplets. Dissection of this domain of TGH and further understanding of how TGH engages a substrate are current topics of investigation.

In addition to the liver, TGH is expressed in other tissues such as the adipose, intestine, kidney and heart (Dolinsky *et al.*, 2004b). The role of TGH in adipose tissue appears to be in basal, rather than hormone stimulated, TG lipolysis (Soni *et al.*, 2004). The role of TGH in other tissues remains speculative and required further investigation. The presence of TGH in the small intestine suggests it plays a similar role in chylomicron production as it does in the liver for VLDL assembly. The function of TGH in kidney and heart remains unclear, although these tissues harbour sparing quantities of TG. It is interesting to note that apoB is expressed in the heart, which can also secrete small, dense apoB containing particles (Boren *et al.*, 1998). This observation begs the speculation that TGH is metabolically linked to apoB in yet undefined ways. The observation that these two proteins can be cross-linked (demonstrated in Chapter 5) is in support of this speculation.

Our laboratory has very recently generated genetically modified mice as models to advance our understanding of TGH. A human TGH transgenic mouse has been created, and initial characterization of these animals is in progress. Protein expression is driven by the metallothionein promoter, which is inducible by inclusion of heavy metal ions in the diet (Searle *et al.*, 1984). Higher levels of TGH in liver are predicted by expression of the transgene, likely providing increased ability to deplete hepatic TG stores. If lipolytic products are channelled into the secretory pathway, TGH over-expression would result in increased VLDL secretion as observed when TGH was over-expressed in McArdle RH7777 cells (Gilham *et al.*, 2005; Lehner and Vance, 1999). Increased plasma VLDL implies susceptibility of the animal to atherosclerosis. Conversely, the lipolytic products could simply be recycled to the storage pool without a net change in hepatic or plasma TG levels. One does not expect significant expression of the transgene in adipose tissue due to the choice of the promoter, and therefore increased free fatty acid release from this tissue is not predicted but will be investigated. The expression levels of the transgene in these animals have been less than anticipated in preliminary experiments, however, the animals have shown trends of higher TG and apoB levels in plasma (E. Wei, unpublished results).

A *Tgh* gene disrupted mouse is also being generated. Heterozygous TGH null mice survive to adulthood, and breeding to generate homozygous mice is currently underway. Because TGH is not expressed in the liver until approximately the suckling / weaning transition (Douglas *et al.*, 2001), survival of the gene disrupted animals is likely as the protein is not present during development. An abundance of experiments with this animal are proposed and will become the object of scrutiny in future directions of the laboratory. The livers of TGH null mice would be expected to have impaired ability to mobilize TG from storage pools, and therefore VLDL associated TG concentrations would be lower in plasma. Data from HSL null mice suggest that fatty acids for hepatic TG are supplied from the adipose tissue (Haemmerle *et al.*, 2002). Because TGH is responsible for basal TG lipolysis in the adipose, hepatic steatosis may not develop in TGH null mice as the fatty acids may be "trapped" in the adipose. Hepatic steatosis may occur if these animals were challenged with a high fat diet. The effects of this gene disruption on the ability of the mice to handle dietary TG and chylomicron formation are exciting avenues of future exploration. The survival of heterozygous TGH null mice also opens up the possibility that humans with mutations in *Tgh* also exist. Ultimately, the transgenic mice overexpressing human TGH and *Tgh* gene disrupted animals will clarify the role of TGH in various physiological processes such as VLDL assembly and operations in extra-hepatic tissue.

One final direction of future research emerges from the numerous examples of enzymes that catalyze the same or similar reactions in lipid metabolism (Vance, 1998). Potentially there are enzymes that can perform a similar function as TGH. A candidate enzyme has already been identified and termed arylacetamide deacetylase (AADA) (Probst *et al.*, 1994). This enzyme if found in the liver, shares homology with HSL and carries the GXSXG motif found in all lipases. Lipolytic activity of AADA has not been tested, however, expression of the AADA cDNA in HepG2 cells increased secretion of free fatty acids and TG (Gibbons *et al.*, 2000). Further studies are required to establish the role of AADA in hepatic glycerolipid metabolism since it was previously

thought that the enzyme's primary role was to eliminate carcinogenic esters (Probst *et al.*, 1994). Ongoing investigations in our laboratory address whether other hepatic carboxylesterases and AADA have the ability to hydrolyze intracellular stored lipids, resulting in increased lipid secretion.

In conclusion, experiments outlined in this Thesis describe significant advances in our understanding of the role of TGH in VLDL assembly and, rationalize this enzyme as a target to combat human diseases including cardiovascular disease (reduced VLDL production), obesity (reduced fat absorption) and Type 2 diabetes (lipotoxicity induced by excessive adipose tissue TG lipolysis and fatty acid export). In addition, numerous aspects of the cell biology and regulation of this enzyme have been characterized. This work also provides a strong foundation for future studies and diverse avenues of investigation. New roles for TGH may be uncovered, especially in extra-hepatic tissues where detailed studies on TGH remain unexplored.

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